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A SIMPLE ELECTROMETRIC COMPARATOR FOR THE DETERMINATION OF HYDROGEN ION CONCENTRATIONS.

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IN an earlier paper by F. W. Gowlett and the writer (1927), an apparatus was described by means of which it was possible to obtain direct readings of pH values. This apparatus was of the thermionic valve type first introduced by Goode (1922), and depended on the special properties of a four-electrode valve.

The apparatus to be described in this paper is based on a different principle.

Both types of apparatus were devised in an attempt to replace the colorimetric method of pH determination by an electrometric method having the advantage of simplicity and rapidity. That the colorimetric method is liable to serious errors where solutions containing proteins are used has been shown, amongst others, by Sørensen (1910, 1912), Homer (1917), and Lepper and Martin (1927). Similarly, in the case of culture media, difficulties are encountered with this method at certain pH ranges. Further, the varying shades of colour in material of this nature make the use of a comparator method essential where any degree of accuracy is desired, while the need for a large number of standard buffer solutions adds to the complication of the method. Thus where a large number of pH determinations have to be made as a routine on sera and culture media, the colorimetric method cannot be considered either as a simple or a very accurate method.

THEORY OF THE APPARATUS.

The principle employed in this apparatus is as follows: An E.M.F. source may be used to charge a condenser, the condenser being subsequently discharged through a ballistic galvanometer; the swing of the galvanometer shows the extent to which the condenser was charged by the E.M.F., and hence a direct relationship exists between the E.M.F. and the galvanometer swing. This of course is well known, and no new basic principle is claimed, but as far as the

writer is aware the method has not been utilized in the manner to be described in this paper.

Until recently it has been customary to use some form of the calomel half-cell in conjunction with the hydrogen electrode, but the introduction of the Quinhydrone electrode by Biilmann (1921) and the subsequent use of a solution containing 0·09 N KCl and 0·01 N HCl, saturated with quinhydrone, as a standard half-cell by Veibel (1923) has simplified the determination of hydrogen ion concentrations. In the case of the calomel half-cells there exists at pH 0 what may be termed a residual E.M.F., *e. g.* at a temperature of 18° C. using the saturated calomel half-cell against a liquid normal in hydrogen ions there would be an E.M.F. of 0·2503 volt. For the working of the apparatus to be described this would be undesirable.

With Veibel's standard half-cell, since the liquid has a pH of 2·04 the pH of the unknown in the cell—

Pt.	0·09 KCl 0·01 HCl Quinhydrone	Saturated KCl	Solution of unknown pH quinhydrone	Pt.

will be given by the equation—

$$\text{pH} = 2{\cdot}04 + \frac{\text{E}}{0{\cdot}0001983\ \text{T.}}$$

Thus where the pH of the unknown differs from the standard by 1 pH unit, the E.M.F. will be +0·0001983 T volt in respect to the standard half-cell if the unknown has a higher pH than the standard, and −0·0001983 T volt if the unknown has a lower pH than the standard. At a temperature of 18° C. the value of 0·0001983 T = 0·0577 volt, and Table I shows the E.M.F. corresponding to the pH range 2–9 which is suitable for use with the quinhydrone electrode system. These values are used for calibrating a scale to read directly in pH; provided the temperature of the electrode system is kept to 18 ± 1° C. results accurate to ± 0·02 pH unit at the high end of the scale will be obtained. At the lower end of the scale the influence of temperature variation is less marked. This system obviates the residual E.M.F. experienced with the calomel cells and is suitable for the present apparatus. In a similar manner the Veibel standard solution (without quinhydrone) may be used as the standard buffer for the double hydrogen electrode described by Cole (1926). The E.M.F. range is the same as that for the quinhydrone electrode system, and where pH values at the higher end of the range are not required with an accuracy greater than ± 0·05 pH unit, the direct reading scale employed with the quinhydrone system may be extended to cover the full pH range from 2–14. (The temperature should be maintained at 18 ± 1° C.)

For more accurate working a scale giving readings in millivolts is to be preferred. At first sight it might appear that this would offer little advantage over the usual method employing a potentiometer to balance the unknown E.M.F., but once the scale has been calibrated the instrument gives directly the value of the unknown E.M.F. It eliminates the time required for the preliminary adjustment of the potentiometer against the standard Weston cell, the balancing of the unknown potential, and moreover, there is no risk of damaging the galvanometer.

In using a ballistic galvanometer it is advisable that the swings should be made in one direction only, and that the instrument should be damped immediately after taking the reading. If attention is paid to these two points it will be found that the "zero" remains practically constant. For biological work it is seldom necessary to go below pH 2, but if readings below this value have to be made the cell connection should be reversed.

TABLE I.—*Showing the E.M.F. to be applied from the Potentiometer when Calibrating the Scale giving Direct Readings in pH. Since the Standard Buffer* (0·01 NHCl + 0·09 NKCl) *has a pH value of* 2·04, *the dividing line of the light-spot should either be set at* 2·04 *on the scale, or set at* 2·0 *and* 0·04 *pH added to every reading greater than* 2·00.

E.M.F.	Corresponding pH at 18° C.	E.M.F.	Corresponding pH at 18° C.
0·0000 volt	2	0·4039 volt	9
0·0577 „	3	0·4616 „	10
0·1154 „	4	0·5193 „	11
0·1731 „	5	0·5770 „	12
0·2308 „	6	0·6347 „	13
0·2885 „	7	0·6924 „	14
0·3462 „	8		

CONSTRUCTION OF THE APPARATUS.

The apparatus is shown diagrammatically in Fig. 1.

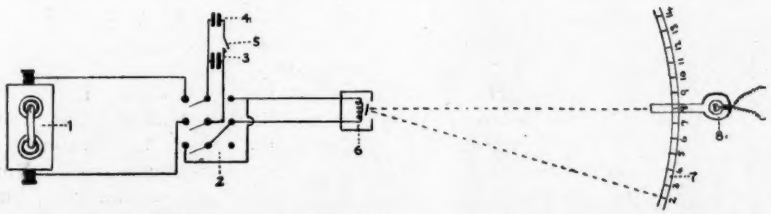

FIG. 1.—Showing the apparatus diagrammatically. 1. Quinhydrone or hydrogen cell. 2. Nine-pole switch (one terminal left blank). 3. 2-mfd. condenser. 4. 1-mfd. condenser. 5. Connecting switch for 1-mfd. condenser. 6. Ballistic galvanometer. 7. Scale calibrated to read pH units direct or in millivolts. 8. Galvanometer lamp.

The galvanometer used is one of the "Student Series" by Pye of Cambridge, giving a swing of 300 mm. per micro coulomb at 1 metre. The relation between the swing of the light-spot in mm. per pH unit and the capacity of the condenser used is as follows:

One volt applied to a condenser of 1 microfarad charges it to the extent of 1 micro coulomb, and on discharging the condenser through the galvanometer a swing of 300 mm. is produced. For 1 pH unit at 18° C. the E.M.F. is 57·7 milli-volts, so the swing will be 17·3 mm. For general use the writer has adopted a 2 microfarad condenser and a working distance of a little over 1

metre, and thus obtained a swing of about 40 mm. per pH. unit. For more accurate work a 3 mfd. condenser giving a swing of about 60 mm. is used.

The condensers used are of the "Mansbridge type," tested to stand a "peak voltage" of 600 volts. These have proved very satisfactory, and hold their charge for a period in excess of that required for correct working of the apparatus.

The switch employed is a 9-pole double-throw pattern. It is essential that contact with the points on one side should be broken completely before contact is made with those on the other side, and that the points should be self-cleaning. The writer has found the "Utility" switch sold for wireless sets very satisfactory.

The lamp and scale stand can be more or less standard and need little comment. For convenience, however, the writer has arranged the galvanometer and lamp on a wall bracket one metre above a bench; the light from the galvanometer mirror is received on a rectangular mirror inclined at an angle of about 45°, from which it passes through a rectangular hole cut in the wall bracket to the scale which lies on the bench. The lamp is placed about 40 cm. and the mirror 15 cm. from the galvanometer. A flat scale was used having a small movement from left to right to enable the zero to be set, and a greater movement backwards and forwards to allow for different scales being brought under the light-spot. The light-spot is observed from the upper surface of the scale with this arrangement.

The light-spot should have a fine dividing line for ease of reading and we have employed for this purpose a fine glass fibre. For details of the preparation of suitable glass fibre the writer is indebted to Miss E. M. Boock. The method is as follows: A length of No. 606 glass tubing is heated in the flame and pulled into a fine capillary, a short piece of which is held by each end, about 1 in. above the small bye-pass light of a Bunsen burner, strong tension being applied. A very fine fibre produced in this manner was cemented across the diaphragm of the light tube and sharply focused on to the scale.

It will be seen that actually only 4 poles of the switch are used to connect the condenser to either the E.M.F. source or to the galvanometer, one of the extra poles being used to damp the galvanometer and the other left blank. For the purpose of damping, the terminals of the galvanometer are short-circuited, no special resistance being employed. While the condenser is connected to the E.M.F. source the galvanometer is damped, and it remains so until the switch is moved to discharge the condenser through the galvanometer.

METHOD OF CALIBRATION.

Since the swings are to be made in one direction only, the torsion head of the galvanometer should be turned so that the resting position of the mirror is out of the central line and the light is reflected to the left of the scale. The leads from a potentiometer should now be connected to the terminals marked 1 in Fig. 1 in place of the cell. The potentiometer is then standardized against a Weston cell in the following manner: Break one lead from the potentiometer and introduce the Weston cell so that its potential is opposed to that of the potentiometer. Set the potentiometer to read 1·0183 volt, and adjust the

accumulator rheostat until no deflection of the light-spot is produced when the switch is moved over and the condenser discharged through the galvanometer. When the potentiometer has been standardized remove the Weston cell, and determine whether on applying a small E.M.F. from the potentiometer the light-spot moves to the right; if necessary reverse the potentiometer leads at the E.M.F. source.

The following method has been found satisfactory for determining the deflection produced by a given E.M.F.: A scale calibrated in millimetres is placed under the light-spot so that the dividing line corresponds with the zero on the millimetre scale. If, now, a given E.M.F. is applied from the potentiometer the length of the swing can be determined in millimetres. A fine needle and a hand lens are of use for determining the exact position of the limit of the swing. By applying the E.M.F. values shown in Table I the relation between E.M.F. and throw in millimetres can be obtained, and scales constructed to read directly in pH for a definite temperature or in millivolts.

It will be found convenient to divide the pH intervals into divisions, each of which is equal to 0·05 pH unit for the 2-mfd. condenser scale and 0·02 pH unit for the 3-mfd. condenser scale.

From observations made during the calibration of this apparatus, it would appear that although a flat scale is used, the variations in length of successive pH intervals is very small. This suggested an alternative method of calibration for use where no potentiometer is available and a high order of accuracy is not required. The method is as follows: Set up a quinhydrone cell with the 0·09 KCl 0·01 HCl mixture (saturated with quinhydrone) against a pH 8·0 buffer (also saturated with quinhydrone) and measure the swing length in millimetres, the cell being kept at 18°C. or such other temperature as may be required. The difference in pH between the two liquids is 8·0–2·04 = 5·96, and from the length of swing obtained from this pH difference, that for a 1·0 pH unit interval can be calculated. Further, it is now possible to obtain galvanometer scales with a curvature of 1 metre radius which should obviate any variation in the length of successive pH intervals.

The buffer solutions used for this purpose should, if possible, be checked electrometrically, but with care they can be prepared with sufficient accuracy. Kolthoff (1925) has described a series of buffer solutions which may be prepared from weighed salts and acids, of which those containing 0·1 M. KH_2PO_4 and 0·05 M. borax in varying proportions would appear particularly suitable for the above purpose. With these reagents buffer solutions having pH values of 6·0, 7·0 and 8·0 may be prepared, and thus three points on the scale in relation to the 0·01 HCl + 0·09 KCl solution established.

The following observations, made while testing the apparatus, are of interest:

The resistance of the quinhydrone cell was artificially increased to over 2,000,000 ohms without effect on the readings obtained. This is a point of great importance when working with liquids having a low electrolyte content.

With a pH 6 buffer as the unknown in a quinhydrone cell whose resistance was about 4000 ohms it was found that the 3-mfd. condenser was completely charged in less than 2 seconds. This time is considerably less than that

required for the light-spot of the galvanometer to become steady after each reading.

Several consecutive readings may be taken, all of which agree, when the quinhydrone cell shown in Fig. 2 is used. This shows that in spite of the small size of the cell, the current drawn from the cell to charge the condenser does not effect its E.M.F. to an appreciable extent.

THE QUINHYDRONE ELECTRODE.

On account of its simplicity the quinhydrone electrode system previously described (Pope and Gowlett) has been retained with slight modification for use with this apparatus. It consists of an ebonite base with two holes containing mercury from which connection is made to two terminals. The electrode tubes consist of a 55 mm. length of No. 606 glass tubing (internal diameter 6 mm.), into one end of which is sealed a platinum wire, either coiled into a spiral or carrying a piece of platinum foil (12 mm. × 5 mm.). "Monax" glass tubing was used as it is a resistance glass; it was found to be more easy to seal the rather heavy gauge platinum wire into this glass than into some varieties. The connecting bridge is a U tube filled with agar saturated with KCl. The construction is shown in Fig. 2.

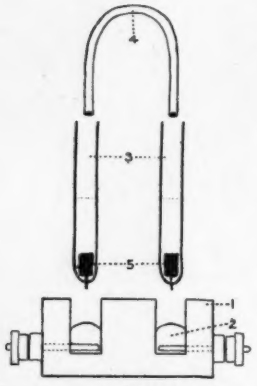

FIG. 2.—1. Ebonite base. 2. Mercury contact to terminals. 3. Electrode tubes. 4. Agar KCl bridge. 5. Platinum foil.

This type of electrode system is easy to make, and has the advantage that it allows of rapid manipulation, and requires only from 0·5–1·0 c.c. of the material whose pH is to be determined. It is equally suitable for either the hydroquinhydrone or the quinoquinhydrone electrodes introduced by Biilmann and Lund (1921).

SUMMARY.

A simple apparatus for the electrometric determination of pH is described, in which the E.M.F. to be measured is applied to a condenser which is subsequently discharged through a ballistic galvanometer.

For a given temperature a scale giving direct readings of pH values may be constructed, or for more accurate work the scale may be calibrated to read directly in millivolts.

With the apparatus, pH values may be determined with an error of 0·02 pH unit or less on material unsuitable for colorimetric methods, the time required being less than that for the colorimetric method.

The apparatus is suitable for use with electrode systems of high internal resistance, *e. g.* for determining the pH of solutions of low electrolyte content. (Attempts to use glass electrodes with this apparatus have, however, proved unsuccessful owing to their extremely high internal resistance (50,000,000-100,000,000 ohms)).

A simple form of quinhydrone electrode is described.

I wish to express my thanks to Dr. J. W. Trevan for his interest, and to Miss M. Llewellyn Smith for her assistance with the calibration of the apparatus and the construction of the scales.

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ON THE INFLUENCE OF TEMPERATURE ON THE RATE OF AGGLUTINATION OF BACTERIA.

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THE agglutination reaction as we do it comprises two quite different processes—firstly the union of the agglutinin of the serum with the bacteria, and secondly the clumping of the bacteria. The work of Gaehtgens (1906, 1908) and Gates (1922) on rapid agglutination by means of centrifuging—work which I have confirmed—has shown that the first process, the union of the agglutinin and the bacterium, takes place very rapidly, and that by far the greater portion of the time in the ordinary agglutination reaction is occupied by the sensitized bacteria coming together into clumps. This is clearly shown in the following experiments:

Experiment 1.—Serial dilutions of a paratyphoid B serum were made and a suspension of paratyphoid *B. bacilli* was added to each. Portions of these were centrifuged immediately and after being incubated for different times with the following result:

	Dilutions of serum.					Control.
	1/200.	1/400.	1/800.	1/1600.	1/3200.	
Centrifuged immediately .	+++	+++	++	+	0	0
,, after 20 minutes	+++	+++	++	+	0	0
,, ,, 2 hours .	+++	+++	++	+	0	0

Experiment 2.—Serial dilutions of the serum of a patient suffering from Malta fever were made and a suspension of *M. melitensis* added to each. A portion of the mixture was centrifuged immediately and the rest was incubated at 48°C. for 6 hours. The following readings were then made:

	Dilutions of serum.					Control.
	1/1250.	1/2500.	1/5000.	1/10,000.	1/20,000.	
Centrifuged immediately .	+++	++	++	+	0	0
Incubated at 48° C. for 6 hours	++	++	+	0	0	0

Gates, working with meningococcus and *B. typhosus*, has shown that the temperature makes some difference to the rate of union of the agglutinin with the bacterium, and he calculates that the time and temperature relations are, in regard to meningococcus, roughly those of chemical acceleration.

In this communication I am not, however, concerned so much with the rate of union of the agglutinin as with the influence of temperature on the rate of flocculation.

It is the experience of everyone who has done agglutination tests that the process is hastened by increasing the temperature up to somewhere about 55°C. The following quotation from the report of a Committee of the Medical Research Council on Pathological Methods may be taken to sum up the matter: "It occurs more rapidly as the temperature rises up to an optimum of 55° C., above which the reaction is impaired and soon ceases. It will take place perfectly at room temperature, but requires a much longer time to become manifest."

It is well known that gentle motion accelerates the flocculation and many devices have been introduced to make use of this. It is probable that the virtue of half-immersion in a water-bath lies in the convection currents which are set up.

That motion is more important than temperature has been shown in many ways; simple rocking of serum bacteria mixtures on a slide at room temperature is a method which has been much practised for obtaining rapid agglutination, while the following experiment brings this point out very clearly.

Experiment 3.—Serial dilutions of a patient's serum were made, and to each dilution an equivalent quantity of staphylococcus suspension was added. Two c.c. quantities of each of these mixtures were placed in two series of tubes, one series being placed in a water-bath at 45°C. and the other connected up with a mixing machine at room temperature. At the end of one hour the tubes gave the following readings:

	Dilutions of serum.				Control.
	1/40.	1/80.	1/160.	1/320.	
Mixing machine at 18° C.	+++	+++	++	+	0
Water-bath at 45° C. .	±	0	0	0	0

The mixing machine described by Dr. Ridley (1928) has furnished a convenient method of doing agglutination tests simultaneously at different temperatures, while at the same time giving the same amount of movement to the fluid in each of the tubes. The movement communicated by the mixing machine is so much greater than any convection currents which may be set up that the latter can be neglected, and for practical purposes we may assume that the movement in all the tubes is equal. With the aid of the mixing machine the following experiments were made to determine whether there was any difference in the rate of flocculation at different temperatures when the fluid in the different tubes was moving equally.

Experiment 4.—Serial dilutions of an agglutinating serum were made and to these equivalent quantities of bacterial suspension were added. These were allowed to remain on the bench for 10 minutes to allow of the union of the agglutinin and the bacteria, after which 2 c.c. quantities were transferred to small tubes and connected with the mixing machine, one series being in a water-bath and another series being at room temperature.

Series 1.—Organism used—staphylococcus. Patient's serum.

	Dilutions of serum.							Control.
	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	
Bench temperature	++	+	0	0	0	—	—	0
Water-bath at 45° C.	++	+	0	0	0	—	—	0
Time, 10 minutes.								
Bench temperature	++	++	++	++	++	+	0	0
Water-bath at 45° C.	++	++	++	++	++	+	0	0
Time, 2 hours.								

Series 2.—Typhoid bacilli. Immune rabbit's serum.

	Dilutions of serum.						Control.
	1/5000.	1/7500.	1/10,000.	1/15,000.	1/20,000.	1/30,000.	1/40,000.	
18° C.	++	+	+	0	0	—	—	0
55° C.	++	++	+	0	0	—	—	0
Time, 15 minutes.								
18° C.	+++	+++	+++	+++	++	+	0	0
55° C.	+++	+++	+++	+++	++	+	0	0
Time, 1 hour.								

A considerable number of experiments have been done with similar results, and it seems clear that a difference of temperature between 18°C. and 55°C. has no influence on the rate of flocculation of the bacteria. There is, however, some difference when flocculation takes place at very low temperatures, as is shown in the following experiment:

Experiment 5.—The technique used was the same as in the previous experiments, except that one set of tubes were immersed in an ice-bath. Typhoid serum and bacilli were used.

	Dilutions of serum.				Control.
	1/500.	1/1000.	1/2000.	1/4000.	
In ice .	+++	+	0	0	0
At 45° C. .	+++	+++	+	0	0

Time, 1 hour.

It is of some interest to record the result of an experiment where the agglutination titre of a serum was estimated by different methods.

Experiment 6.—Serial dilutions of an anti-pneumococcus serum were made and to these were added equal quantities of a suspension of pneumococcus. These were allowed to remain on the bench for 5 minutes to allow of the union of the agglutinin with the bacterium; they were then treated as under with the following results:

	Dilutions of serum.					Control.
	1/20.	1/80.	1/160.	1/320.	1/640.	
Centrifuged for 5 minutes .	+++	+++	+++	±	0	0
1¼ hours kept in motion at room temperature with mixing machine	+++	++	+	0	0	0
1¼ hours at 45° C., half immersed, stationary	+	0	0	0	0	0
3 hours at 45° C., half immersed, stationary	++	±	0	0	0	0

It will be seen that with an organism like pneumococcus, which does not agglutinate very rapidly, one obtains the highest titre after centrifuging for 5 minutes; next comes gentle movement at room temperature, and last of all comes the ordinary method of incubation at a high temperature in a water-bath with the tubes half-immersed. Of course, if the time of incubation had been prolonged to 18 or 24 hours the same titre would probably have been obtained, but an experiment such as this shows the great advantage of the centrifuge method in obtaining satisfactory results in a short time.

CONCLUSIONS.

In agglutination tests, while the rate of union of the agglutinin with the bacterium is hastened to some extent by a rise of temperature (Gates, 1922), the rate of flocculation is not affected by temperature changes between 18°C. and 55°C. There is, however, some delay at temperatures just above freezing-point. It follows, therefore, that the increase in the rate of flocculation with

increase in the temperature when agglutination tests are done in the ordinary way is due to convection currents which are set up in the fluid in the different tubes. These currents would be more marked at the higher temperatures, thus bringing the bacteria more rapidly into contact with each other and so favouring flocculation.

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OBSERVATIONS ON THE MODE OF ACTION OF A VIRICIDAL SERUM.

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IT is a well-established fact that the filtrable viruses are capable of giving rise to specific neutralizing antibodies, but the nature and mode of action of these antibodies still remains somewhat obscure. Although there is some evidence, in the case of a rabicidal serum at any rate (Murillo, 1911; Kondo, 1922), that the time and temperature to which the mixtures of virus and serum are exposed are important factors in neutralization experiments, this is not generally conceded and there are those who hold that no interaction takes place between neutralizing antibody and virus *in vitro*, the viricidal phenomenon having as its essential setting the living tissues or the interior of living cells. Such a conception implies a fundamental difference between the mode of action of the neutralizing antibody of a viricidal serum and those antibodies with which we are familiar in bacteriological work. There are, of course, considerable difficulties inherent in the demonstration of what actually takes place when a virus is mixed with its specific viricidal serum. One cannot separate the two as one could in an absorption experiment with one of the visible bacteria, so that this method is not available for determining whether a filtrable virus unites with its neutralizing antibody in the test-tube.

However the same difficulty must have presented itself in the earlier work with toxin and antitoxin and had it not been for the classical experiment of Martin and Cherry it would probably have been thought that union between toxin and antitoxin did not occur *in vitro*. There is considerable evidence that specific complement-fixation can be obtained with many of the filtrable viruses and their corresponding antisera, though the specificity of these reactions has been called in question by Schultz and his co-workers (Schultz, 1928; Schultz, Bullock and Lawrence, 1928; Schultz, Bullock and Brewer, 1928), and although it is recognized that neutralizing antibody and complement-fixing antibody are not necessarily identical, the results obtained with the complement-fixation reaction encourages the belief that virus and neutralizing antibody do unite *in vitro*. The following experiments have been carried out to ascertain whether this is so or not.

Working Hypothesis.

It was thought that if one took a mixture of virus and antiserum just neutral as tested *in vivo*, and in which the virus concentration was considerable, on making dilutions of this mixture one might rapidly pass beyond the effective range of the serum while still being within that of the virus. In this manner one might be able to determine the state of the virus in an apparently neutralized mixture and so ascertain whether any interaction between virus and antibody was taking place in the test-tube. At first sight this conception might appear unreasonable because dilution of a balanced mixture of virus and antibody reduces both these two factors in the same proportion. But the rate at which antigen and antibody unite depends on their concentration so that by diluting a balanced mixture of virus and antiserum the time required for their interaction is prolonged. Should the union between virus and neutralizing antibody take place in the tissues then a mixture of the two would only fail to give a take when the time required for their union was less than that required for the virus to establish itself. So by the simple procedure of dilution and consequent prolongation of the time required for the serum to act, one should be able to unmask active virus in an apparently neutralized mixture.

Experimental.

Virus.—The virus employed in this work was a guinea-pig skin strain of herpes febrilis which was obtained in the first instance from labial vesicles in a case of lobar pneumonia. It has been passed in guinea-pigs for seven months, and has behaved throughout with great regularity. Each sample of virus worked with was titrated as described in a previous communication (Bedson and Crawford, 1927).

Sera.—Two antiherpes sera have been employed; B. 369, a hyperimmune serum prepared in the guinea-pig and "S" obtained from a guinea-pig six weeks after inoculation with herpes virus. Both sera were inactivated by heating for 30 minutes at 56°C. The titre of the sera was ascertained by mixing falling concentrations of serum and constant quantities of virus in equal parts and inoculating the mixtures by scarification on the epilated skin of

guinea-pigs. The mixtures were allowed to stand for 30 minutes at room temperature before inoculation, and the dilution of virus employed in the test represented 100 M.I.D. The titre of the serum represents the highest dilution of the serum capable of neutralizing this given quantity of virus under these test conditions.

Experiment 1.

Herpes virus B. 694—a phosphate diffusate—titre 1 in 100 was mixed undiluted in equal parts with an antiherpes serum B. 369 diluted 1 in 50 =A. 1. The final concentrations of virus and serum in this mixture A. 1 were therefore 1 in 2 and 1 in 100 respectively, and from previous observations these relative proportions of the two should give neutralization or almost complete neutralization. A five-fold dilution of this mixture would contain virus diluted 1 in 10 and serum diluted 1 in 500 and a fifty-fold dilution virus 1 in 100 and serum 1 in 5000. Mixtures containing these relative proportions of serum and virus were also made; A. 2 (virus 1 in 10 and serum 1 in 500) and A. 3 (virus 1 in 100 and serum 1 in 5000). After standing for 30 minutes at room temperature the presence of active virus in the different mixtures was tested for by inoculation in the guinea-pig (scarification) and at the same time five-fold and fifty-fold dilutions of the original mixture A. 1 were made = A. 2* and A. 3* and similarly inoculated in a guinea-pig.

The results of these inoculations are tabulated below (Table I):

TABLE I.

	Dilutions of virus.			
	1/1	1/10	1/100	1/1000
Titration of virus in phosphate	+++	++	± (4)	−
	A. 1.	A. 2.	A. 3.	
Serum virus mixture A. 1, 2 and 3	∓ (1)	++ (20)	∓ (1)	
		A. 2.*	A. 3.*	
Phosphate dilution of A. 1, A. 2,* A. 3*		++ (17)	∓ (1)	

+ + + = Confluent vesicles. + + = Numerous non-confluent vesicles.
+ = Moderate take. ± = 3–6 vesicles.
∓ = 1–3 vesicles. − = No take.

The figures beside the sign indicate the number of vesicles observed.

Clearly the mixture A. 1 is almost completely neutralized whilst those in A. 2 and A. 3 show little difference in virus readings from those obtained in the titration in phosphate. The five- and fifty-fold dilutions of A. 1, viz. A. 2* and A. 3,* give practically identical readings with those of A. 2 and A. 3, showing that a mixture of serum and virus though apparently neutralized after standing 30 minutes at room temperature, contain in reality as much active

virus as it did originally. Thus after this lapse of time at room temperature no fixation of neutralizing antibody seems to have taken place.

Experiment 2.

Virus B. 699. Phosphate diffusate. Titre 1 in 100.
Serum B. 369.

Virus undiluted was mixed with serum diluted 1 in 40 in equal parts. Five- and fifty-fold dilutions were made from the original mixture after 15 minutes and 4 hours at room temperature and virus tested for as before with the following results (Table II):

TABLE II.

Time of test.	Original mixture.	5-fold dilution.	50-fold dilution.
After 15 minutes	++	+++	+
After 4 hours	−	∓ (1)	−

In this experiment the original mixture tested after 15 minutes only showed partial neutralization, nevertheless dilution enables one to demonstrate masked active virus after this lapse of time. It seems quite clear however that by allowing a longer time for the interaction of virus and neutralizing antibody, viz. 4 hours, fixation of antibody has actually taken place.

Experiment 3.

Virus B. 718. Phosphate diffusate. Titre 1 in 100.
Serum B. 369.

The same procedure was observed as in Experiment II with the exception that tests for active virus were made 5 minutes, 1 hour, and 4 hours after making the initial mixture. The results are given below:

TABLE III.

Time of test.	Original mixture.	5-fold dilution.	50-fold dilution.
After 5 minutes	++	++	± (4)
After 1 hour	±	+ (10)	−
After 4 hours	∓ (1)	−	−

Experiment 4.

Virus B. 726. Emulsion of vesicle epithelium. Titre 1 in 10,000.
Serum (S). Titre 1 in 20.

Virus diluted 1 in 100 was mixed with serum diluted 1 in 10 in equal parts and the presence of active virus tested for in the original mixture and five- and fifty-fold dilutions thereof after 5 minutes and 60 minutes at room temperature.

TABLE IV.

Time of test.	Original mixture.	5-fold dilution.	50-fold dilution.
After 5 minutes	∓ (2)	+ (7)	∓ (1)
After 60 minutes	—	—	—

Experiment 5.

Virus B. 733. Phosphate diffusate. Titre 1 in 100.
Sera (*a*) anti-herpes "S." Titre 1 in 20.
(*b*) Normal guinea-pig (inactivated at 56° C.).

This experiment was carried out in order to demonstrate that the inactivation of the herpes virus observed in the foregoing experiments was actually due to the neutralizing power of the antiserum and not merely to some non-specific effect exerted by serum after prolonged contact with the virus at room temperature. Undiluted virus was mixed with equal parts of—

A. Serum "S" diluted 1 in 10.
B. Normal guinea-pig serum diluted 1 in 10.

The presence of active virus was tested for in A and B and five- and fifty-fold dilutions thereof immediately after making the original mixtures and after they had stood for 2 hours at room temperature. The results are recorded below:

TABLE V.

Time of test.	Original mixture A.	5-fold dilution.	50-fold dilution.	Original mixture B.	5-fold dilution.	50-fold dilution.
Immediately after making original mixtures.	± (4)	+ (10)	∓ (1)	+++	++ (20)	∓ (2)
After 2 hours.	—	—	—	+++	Becoming confluent ++	± (5)

Repetition of this experiment with another specimen of normal guinea-pig serum gave a similar result so that it is obvious that the inactivation of the virus is a specific effect. The evidence adduced warrants the conclusion that virus and neutralizing antibody do unite *in vitro* but, of course, throws no light on what actually happens to the virus outside the animal body. Whether the virus is actually rendered inert in the test-tube or whether it is only prepared for phagocytosis or lysis in the tissues of the animal remains to be determined. Gordon (1925) obtained evidence in one experiment with vaccinia virus which pointed to complement playing a part in the neutralization of the virus *in vitro*, but further work on this point is required. To argue from the results obtained with herpes virus to other viruses is perhaps not quite legitimate, but probably the same holds good in their case also. There does not appear to be any reason for supposing that viricidal sera differ fundamentally in their mode of action from that of an anti-bacterial serum and the phenomena observed in the study of immunity in reference to bacteria find a close parallel in what is being brought to light in the case of filtrable

viruses. As our knowledge of this group of pathogenic agents increases their resemblance to the cultivable bacteria becomes greater and it becomes increasingly difficult to find a group characteristic which can be accurately described as differentiating between the two.

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ON THE DIVISION OF CELLS UNDER VARYING TENSIONS OF CARBON DIOXIDE.

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IN a recent paper (Mottram, 1927) experiments were described showing that both normal cells and sarcoma-cells were extruded from *in vitro* tissue cultures at tensions of carbon dioxide far above the 40 mm. naturally found in normal tissues. In the same paper evidence was brought forward that an increased carbon dioxide tension in the tissues may be an important factor in the ætiology of cancer. This communication pursues further the line of investigation, and deals with the effect upon cell division of varying tensions of carbon dioxide.

The kidneys of young rats of approximately 50 gm. were cultivated, and the cell division of kidney-cells and of fibroblasts was observed. The cultures were made in chicken plasma 2 parts, and rat embryo extract 1 part. Hollow-ground slides were used having grooves cut so that there was free communication with the outside. The cultures were placed in Petri dishes of which the bottom was covered with blotting-paper soaked with 4 c.c. of Locke's solution. The Petri dishes were placed in McIntosh Fildes anaerobic jars. Carbon dioxide was introduced into the jars by first exhausting them to a known pressure and then filling up with CO_2. The percentage of CO_2 was also

ascertained during, and at the end of the experiment, by analysis using a Haldane's apparatus. Incubation was carried out at 36° C. for 3 or 4 days.

It was found that different samples of rat kidney, of plasma or of embryo extract gave varying results, so that all the materials used must be identical, allowing only the CO_2 tension to vary, in order to obtain comparable results. The experiments were therefore carried out in pairs; for instance, 12 cultures were placed in a jar containing 50 mm. CO_2, and 12 in a jar containing 20 mm. CO_2. They were filled at a room temperature of 18·5° C., and the barometer was 767 mm. After 3 days the analysis using Haldane's apparatus gave at 20 mm. 2·9% and 3·1% of CO_2; at 50 mm. 6·0% and 6·1% of CO_2. The barometer was 770 mm. The cultures were removed, fixed in Bouin's solution and stained with Weigert's iron-hæmotoxylin. In each slide the number of mitoses in not more than 300 cells was counted for both fibroblasts and kidney-cells. In this way 20 experiments in pairs have been carried out, with the results shown in the accompanying table.

From this table it is possible to deduce approximately the optimum tension of CO_2 for the cell division of normal cells. For instance, Experiment 5 shows that it is nearer 50 mm. than 10 mm., and Experiment 16 nearer 50 than 80 mm. Therefore it must lie somewhere between 10 and 80, but nearer to 50 mm. than to either of these tensions. Comparing Experiments 16 and 17, it is clear that it lies below 50. It is therefore not far removed from 40 mm., which is the tension of CO_2 found in normal living tissue. It may be pointed out that the two lowest figures for fibroblasts, 24 and 23, were observed at 40 mm. tension (Experiments Nos. 19 and 18), and the two lowest for kidney-cells 54 and 56 at 40 and 30 mm. (Experiments Nos. 18 and 6).

The table also shows that cell division occurs at tensions of CO_2 far above (200 mm.) the normal CO_2 tension (40 mm.) of the tissues, and also far below (0 mm.).

Whilst counting these mitoses of fibroblasts many abnormal figures were observed in the cultures grown at high tensions. The departure from the normal consisted in an irregular migration of the chromatin towards the centrosomes; some chromatin remained suspended at the equator of the spindle, whilst other fragments had already migrated to the centrosome. This unusual arrangement was more often than not asymmetrical, a fragment of chromation being present at one centrosome with none at the other, or more fragments at one centrosome than at the other. These appearances are shown in Fig. 1.

It was also observed that the size of the nuclei of undividing cells under high tension of CO_2 was increased, and reduced at low tensions, as compared with nuclei of cells at 40 mm. of CO_2.

The results show that normal cells divide most freely when the cultures are kept under tensions of CO_2 not far removed from the tension (40 mm.) found in normal tissues. Further, they show that cell division occurs at tensions far above (200 mm.) and far below (0 mm.) this level; and that at high tensions abnormal mitoses occur, in which there is an irregular migration of chromatin to the centrosomes. This irregularity is similar to that which is seen in cells which have been subjected to X-radiation (Mottram,

	Fibroblasts.				Kidney-cells.			Fibroblasts.				Kidney-cells.		
	Pressure of Co_2 in mm.	Number of cells counted.	Number of mitoses.	Proportion.	Number of cells counted.	Number of mitoses.	Proportion.	Pressure of Co_2 in mm.	Number of cells counted.	Number of mitoses.	Proportion.	Number of cells counted.	Number of mitoses.	Proportion.
1	50	1004	7	143:1	...	N.C.	...	200	...	No mitoses	...	...	No mitoses	...
2	25	1714	26	66:1	1400	5	282:1	100	514	6	86:1	...	No mitoses	...
3	70	2000	10	200:1	474	6	79:1	15	3000	10	300:1	2164	8	271:1
4	20	1482	20	74:1	1141	7	163:1	60	...	No mitoses	...	...	No mitoses	...
5	50	3000	34	38:1	1058	9	117:1	10	1150	9	116:1	1372	5	274:1
6	30	3000	41	73:1	1012	18	56:1	15	3000	29	103:1	1264	5	253:1
7	35	2800	36	78:1	1096	9	122:1	20	2800	38	74:1	1054	5	211:1
8	40	...	N.C.	...	950	6	158:1	25	...	N.C.	...	723	3	241:1
9	50	3609	71	51:1	1735	14	124:1	35	2250	46	49:1	1855	10	185:1
10	45	2431	36	68:1	2095	21	100:1	60	1577	27	58:1	2868	22	130:1
11	55	950	25	38:1	673	4	168:1	75	817	14	58:1	793	5	158:1
12	65	2103	32	66:1	...	N.C.	...	90	2800	32	88:1	...	N.C.	...
13	90	1272	9	141:1	1100	8	138:1	140	1382	9	154:1	1200	8	150:1
14	90	1700	36	48:1	1800	11	164:1	160	1289	19	67:1	1200	4	300:1
15	110	2400	59	41:1	1018	6	170:1	200	2940	10	294:1	3000	10	300:1
16	50	3000	105	29:1	1058	11	96:1	80	2400	45	55:1	906	4	226:1
17	50	2400	82	29:1	2100	30	70:1	20	2700	90	30:1	1500	10	150:1
18	70	3000	115	26:1	1500	24	63:1	40	3300	137	24:1	1200	22	54:1
19	40	3600	155	23:1	...	N.C.	...	10	3600	110	33:1	...	N.C.	...
20	15	3000	61	49:1	...	No mitoses	...	0	1200	8	150:1	...	No mitoses	...

N.C. = insufficient number of cells for a count.

1913), where, besides fragmentation of the chromatin into fine granules, delay in its migration to the centrosomes occurs, so that whilst some chromatin has moved to the centrosomes some remains suspended at the equator of the spindle. This similarity is heightened by the increase and size of the nuclei under high tensions of CO_2, for this also occurs after the radiation of cells (Mottram, 1927). In the case of radiation this appears to be due to an increase in the nuclear hyaloplasm, and it has been suggested that the changes in chromatin are secondary to this nuclear change (Mottram, 1926). Perhaps here also the aberration of the chromatin follows increase in nuclear hyaloplasm; for the reason that an alteration of the pH value of the interior of living cells, which CO_2 is likely to produce more than any other agent, effects changes in the volume of the nuclear hyaloplasm by diffusion through the nuclear membrane.

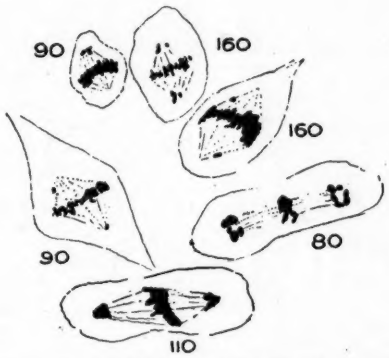

FIG. 1.—Shows abnormal mitoses of fibroblasts cultured at high CO_2 tensions; the numbers give the CO_2 tensions.

These observations are of interest from the point of view of the cancer problem. It has long been in the mind of some workers that cancer following radiation might be due to disturbances in mitoses which radiation is known to produce, and that by unequal division of the chromatin among daughter-cells somatic mutations might arise which would give the cells abnormal characters and throw them out of tune with the normal cells of the body. Boveri (1914) was the first to suggest this "somatic mutation" hypothesis from his work on multipolar cell division in sea-urchin eggs fertilized with two instead of one sperm. Pathological development results, associated with the abnormal distribution of the chromosomes in the primary blastomeres. Until recently there has been no evidence of the production of mutations by radiation. This is, however, now an established fact. Muller (1927), for instance, has shown that the natural occurrence of mutations in drosophila can be increased 15,000% by subjecting the flies to radiation. It seems likely, therefore, that somatic mutations will follow the radiation of normal tissues, and might explain the occurrence of cancer after radiation.

From this point of view the abnormal mitoses occurring in association with high CO_2 tensions suggest that somatic mutations might be caused in the

tissues by an interference with blood-supply (Kreyberg, 1927), so that the hypothesis as an explanation of cancer could cover a wide field and not be confined to cancer following radiation (Mottram, 1928).

CONCLUSIONS.

(1) When normal cells are cultivated *in vitro* under varying tensions of CO_2, mitoses are observed to be most abundant at a CO_2 tension approximating to that of normal living tissues, which is 40 mm.

(2) Mitoses occur over a wide range of CO_2 tension, from 0 to 200 mm.

(3) Under high CO_2 tensions abnormal mitoses occur, the abnormality consisting of an irregular migration of the chromatin to the centrosomes.

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ON A DILUTION PHENOMENON OBSERVED IN THE TITRATION OF THE SERUM OF FOWLS IMMUNIZED AGAINST THE VIRUS OF FOWL PLAGUE.

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In the course of some experiments on the titration of an immune serum prepared for the virus of fowl plague certain observations were made which seem interesting, not only as affecting the general principles of the titration of such sera, but also as possibly throwing some light on the mechanism of the so-called "viricidal" action of immune sera on the ultra-microscopic viruses.

The experiments in question were made with the pooled sera of a number of fowls which had been immunized against fowl plague—either by a phenol glycerine vaccine (Todd, 1928) or by double inoculation with immune serum and virus—and then hyperimmunized by three or four intramuscular injections of the virulent blood of fowls suffering from the disease. The virus against which the serum was titrated was used in the form of virulent citrated blood-plasma. To prepare this two or three fowls were inoculated intramuscularly with the virus, and some 28 hours later were bled to death and the blood citrated and placed in the cold room at 0° C. Next day the blood was centrifuged and the supernatant plasma pipetted off and re-centrifuged. The

resulting clear plasma, which was free from visible particles, was kept frozen at -10° C.

As the infectivity of the blood varies in individual fowls, it was found advisable to pool the plasma of several birds, and in this way plasma killing a fowl at a dose of about 1/100,000 c.c. when given intramuscularly could generally be obtained. The citrated plasma when kept frozen at -10° C. appears to maintain its virulence remarkably well, at any rate for some months, and no appreciable change in the minimal infective dose was observed after three months' storage at this temperature.

In titrating the immune serum against the virus the method originally used was to take a series of test-tubes, each containing 1 c.c. of the immune serum, and to add to each of these an equal volume of varying dilutions of the virulent plasma. After thorough mixing the tubes were placed in a water-bath at 37° C. for a time—commonly 2 hours—and the contents then tested by intramuscular injection into fowls.

With the particular rather weak immune serum used in the earlier experiments it was found that the strength of the serum was such that a mixture of 1 c.c. of the serum with 1 c.c. of a 1 in 100 dilution (*i. e.* 1000 minimal infecting doses) of the virulent plasma gave rise to no symptoms in a fowl injected intramuscularly with 2 c.c. of the mixture.

Now for certain reasons, notably the desire to avoid the complication of passive serum immunity, it was thought advisable to work with smaller quantities of the immune serum and a dose of 0·1 c.c. was selected as a suitable amount, the tests being carried out in exactly the same way with the exception that a tenfold dilution both of the serum and of the dilution of virulent plasma was used—the total volume being 2 c.c. as before.

As 1 c.c. of the serum was known to protect against 1000 minimal infecting doses, it was assumed that one-tenth of this amount (*i. e.* 0·1 c.c.) would probably protect against 100 minimal infecting doses. This was, however, found not to be the case, as fowls receiving the weaker diluted mixture died of acute fowl plague.

An experiment was then made in which two identical mixtures were prepared, each consisting of 0·1 c.c. immune serum, 0·1 c.c. of a 1 in 1000 dilution in saline of citrated virulent plasma (*i. e.* 100 minimal infecting doses).

These mixtures were kept at 37° C. for 2 hours and then injected intramuscularly into two fowls, the only difference being that whilst the first fowl received the mixture without dilution, in the case of the second fowl the mixture was diluted ten times—being made up to 2 c.c.—with saline before injection.

The result was very striking: the fowl which had received the undiluted mixture showed no symptoms, while the bird which had received the diluted mixture died of acute fowl plague. As both fowls had received the same number of minimal infecting doses of the virus together with the same amounts of immune serum, the difference in the results could only be attributed to the different dilutions in the two cases.

This experiment was then repeated, varying the times for which the serum and virus were incubated together and also the conditions of dilution in order to see if these were factors in the production of the phenomenon. A number of these experiments are shown in the following table:

Action of Immune Serum on the Virus in vitro: *Dilution Phenomenon.*

1. *With the Undiluted Mixture.*

Method.—0·1 c.c. of immune serum (No. 31) mixed with 0·1 c.c. (*i. e.* 100 M.I.D.) 1 in 100 dilution of virus (No. 368.9). Mixture injected intramuscularly. Total volume of dose 0·2 c.c.

Fowl No.	Immune serum.	Virus.	Time of contact at 37° C.	Result.
15	0·1 c.c.	100 M.I.D.	2 hours	Lived.
21	,,	,,	1½ ,,	,,
27	,,	,,	2 ,,	,,
30	,,	,,	30 minutes	,,
31	,,	,,	2 hours	,,
32	,,	,,	,,	,,
39	,,	,,	Injected immediately	,,
65	,,	,,	10 minutes	,,

2. *With a Tenfold Dilution of the Mixture in Saline.*

Method as above, except that the mixture was diluted to ten times its original volume with saline before injection. Total volume of dose 2 c.c.

Fowl No.	Immune serum.	Virus.	Time of contact at 37° C.	Diluted.	Result.
385	0·1 c.c.	100 M.I.D.	2 hours	Before mixing	Died (4 days).
16	,,	,,	,,	,,	,,
28	,,	,,	,,	10 min. after mixing	,, (2–3 days).
50	,,	,,	1½ hours	1½ hours after mixing	,, ,,
59	,,	,,	,,	Ditto	,, ,,
66	,,	,,	10 min.	10 min. after mixing	,, ,,

M.I.D. = minimal infecting dose (intramuscular).

Thus, with intramuscular injections of the particular mixture of serum and virus used, the dilution phenomenon took place regularly—the undiluted mixtures being quite harmless, but becoming virulent when diluted to ten times their original volume with saline. Moreover the result in these experiments was the same whether the undiluted serum and virus were mixed and kept for 2 hours at 37° C., and then diluted, or the serum and virus were separately diluted before mixing.

It would appear, however, that this apparent independence of the conditions of dilution and contact in the above experiments may have been due to the particular proportions of the serum and virus used, and must not be taken as generally true, as a good deal of evidence was met with later suggesting that these factors may be of importance. For example, a test was made by mixing a certain volume of virulent plasma with the minimum amount of immune serum necessary to render the mixture non-virulent on intramuscular injection. Samples of this mixture were tested on fowls immediately after mixing (undiluted, diluted tenfold and diluted a hundredfold), and similar tests were

made after the mixture had been kept at 37° C. for 2 and 4 hours. The results were as follows:

Dilution Phenomenon: Influence of Time of Contact and of Dilution.

Method.—Citrated virulent plasma (No. 41) mixed with one-tenth of its volume of immune serum (No. 111), and the mixture kept at 37° C. At varying times samples of 0·1 c.c. taken out and tested intramuscularly on fowls with and without dilution, the dilution being made immediately before injection.

Dose.	Dilution.	Mixed and injected at once.	Kept at 37° C. for 2 hours and then injected.	Kept at 37° C. for 4 hours and then injected.
0·1 c.c.	1/1 (undiluted)	Lived	*Died* (5 days)	Lived.
1·0 c.c.	1/10	*Died* (51 hours)	*Died* (3 days)	*Died* (4 days).
10·0 „	1/100	*Died* (under 44 hours)	*Died* (49 hours)	*Died* „

In this experiment the conditions of contact and dilution appear to have had a definite effect, but a good deal of further work will be required before the exact influence of these factors can be definitely formulated. It will be noticed that in this experiment one of the fowls which had received the undiluted mixture died (after 5 days). Similar irregular results were occasionally encountered and were provisionally attributed to an uneven distribution of the virus in the citrated virulent plasma, as it was noticed that some of the tubes of plasma, after being thawed, showed the presence of fibrin-like flakes suggesting a partial clotting. To eliminate this difficulty in later experiments the dilutions of plasma were centrifuged before use.

When the mixture of immune serum and virus was kept at 28° C. for 24 hours and then diluted with ten times its volume of saline and injected intramuscularly, the dilution phenomenon was no longer observed, but here there is the possibility that some deterioration of the virus may have taken place during the long exposure to this temperature.

In order to determine how small a degree of dilution would produce the dilution phenomenon, a test was made in which the same amounts of the same immune serum and virus as were used in the previous tests were made up at progressively increasing dilutions and then tested intramuscularly on fowls. The results are shown in the following table:

Effect of Degree of Dilution.

Method.—Each of the following fowls received a mixture of 0·1 c.c. of immune serum (No. 31) + 0·1 c.c. (*i.e.* 100 M.I.D.) of a 1 in 100 dilution of virulent citrated plasma (No. 368.9), but the constituents of the mixture were diluted to varying degrees *before* mixing. Mixtures allowed to stand for 1½ hours at 37° C. and then injected intramuscularly.

Fowl No.	Dose.	Result.
21	0·2 c.c. mixture 1/1 (undiluted)	Lived.
22	0·4 „ „ 1/2	„
23	0·8 „ „ 1/4	*Died* (2½ days).
24	1·6 „ „ 1/8	„ (3 „).

Thus, under the conditions of the experiment a dilution of 1 in 4 of the original mixture was sufficient to show the phenomenon. As the virulent plasma used in the original mixture, however, was already diluted to 1 in 100, the 1 in 4 dilution of the original mixture really represents a dilution of approximately 1 in 8 of the serum plasma mixture.

Presumably the degree of dilution required to elicit the dilution phenomenon depends upon the relative proportions of immune serum and virus used, as the phenomenon is only observed with mixtures which are approximately "balanced"; and a mixture which contains any considerable excess of immune serum does not become virulent on dilution. This is shown in the following experiment:

Proportion of Serum to Virus necessary to Extinguish the Dilution Phenomenon.

Method.—The undiluted virulent plasma and immune serum were mixed, diluted with approximately ten times their volume of saline and, after standing for 1½ hours at 37° C., were tested intramuscularly on fowls.

Fowl No.	Virus No. 104.	Immune serum No. 111.	Saline.	Result.
158	0·1 c.c. (10,000 M.I.D.)	0·01 c.c.	1 c.c.	*Died* (2–3 days).
159	„ „	0·02 „	„	„ „
160	„ „	0·03 „	„	Lived.

In this experiment the amount of immune serum (0·01 c.c.) used in the case of the first fowl was the amount which, when mixed with 0·1 c.c. of the virulent plasma, had been found to be just sufficient to render the undiluted mixture harmless to a fowl on intramuscular injection. It will be seen that when this amount of serum was trebled the dilution phenomenon no longer occurred.

In a similar experiment where the undiluted serum and virulent plasma were incubated together at 37°C. for 2 hours before dilution, doubling the amount of serum suppressed the phenomenon.

A most interesting fact in connection with the dilution phenomenon is that if fresh normal fowl serum is used as the diluent in place of saline the phenomenon does not occur. It was at first thought that the viscosity of the serum might be responsible, but experiments with more viscid diluents such as 10% solution of gelatine, 7% solution of soluble starch in saline, and 50% glycerine showed that these solutions behaved like saline and did not affect the phenomenon.

It was further found that the addition of a small amount of a suspension of killed staphylococci to the saline used for the dilution also inhibited the phenomenon. These experiments on the effect of the nature of the diluent are still in an early stage and are being continued.

As there is reason to believe that the virus of fowl plague, as it exists in the blood, is mainly associated with some of the cells of the white cell layer, and is possibly to a large extent actually within these cells (Todd, 1927), it was interesting to see if the dilution phenomenon would occur if the whole blood

were used as a source of the virus in the experiments—in place of the cell-free plasma previously employed. The following experiment shows that the dilution phenomenon takes place equally well when the whole blood is used :

Dilution Phenomenon : Using Whole Blood as a Source of the Virus.

Method.—In this experiment a 1% suspension of the citrated blood of a fowl in the acute stage of the disease was used in place of the cell-free citrated plasma used in previous tests with immune serum (No. 31) :

1. *Using the Undiluted Mixture.*

Fowl No.	Immune serum.	1% susp. vir. blood.	How given.	Result.
39	0·1 c.c.	0·1 c.c.	Mixed and injected at once	Lived.
40	,,	,,	Mixed and injected after 2 hours at 37° C.	,,

2. *Using a Tenfold Dilution of the Mixture in Saline.*

Fowl No.	Tenfold dilution of immune serum.	Tenfold dilution of 1% susp. vir. blood.	How given.	Result.
41	1 c.c.	1 c.c.	Constituents diluted before mixing. Mixture kept 2 hours at 37° C. and then injected.	Died (2½ days).

This experiment was repeated with the whole virulent blood of another fowl with a similar result.

Another interesting experiment was to make the dilution *in vivo* by injecting the undiluted mixture of serum and virus directly into the blood-stream, so that the dilution was made actually in the circulating blood. In this experiment equal parts of the undiluted serum and of a dilution of the virus in saline containing 1000 minimal infecting doses per c.c. were mixed, kept for 2 hours at 37°C., and then tested by the injection of 0·2 c.c. of the mixture intravenously into fowls—(*a*) undiluted, and (*b*) after tenfold dilution with saline (*i.e.* after making up to 2 c.c. with saline) :

Intravenous Injection of Mixtures.

1. *With the Undiluted Mixture.*

Method.—Equal parts of the undiluted serum (No. 31) and of a dilution of the virus (No. 368.9) containing 1000 M.I.D. per c.c. were mixed, kept at 37° C. for 2 hours and then tested by intravenous injection into fowls.

Fowl No.	Dose.	Result.
35	0·2 c.c. mixture (containing 100 M.I.D.)	*Died* (6 days).
45	,, ,, ,, ,,	,, (3–4 ,,).
46	,, ,, ,, ,,	,, (3–4 ,,).

2. *With a Tenfold Dilution of the Mixture in Saline.*

Method.—Equal parts of a 1 in 10 dilution of the serum (No. 31) in saline and of a dilution of the virus (No. 368·9) containing 1000 M.I.D per c.c. were mixed, kept at 37° C. for 2 hours and then tested by intravenous injection into fowls.

Fowl No.	Dose.	Result.
36	2 c.c. mixture (containing 100 M.I.D.)	*Died* (2½ days).
47	„ „ „ „	„ (2–3 „).
48	„ „ „ „	„ („ „).

It will be seen that the undiluted mixture, which was quite harmless when given intramuscularly, gave rise to fowl plague when injected intravenously. Dilution of the mixture before the intravenous injection, however, appeared to render it still more virulent, judging from the dates of death of the respective birds. The question has not yet been investigated, but it appears unlikely that the difference in the results of intravenous and intramuscular injection is simply a dilution effect. It should be mentioned that the fowl, if anything, appears to be rather less sensitive to intravenous than to intramuscular injections of the virus; the difference in any case is small, as in a comparative test a virus which killed a fowl at 1/100,000 c.c., and not at 1/300,000 c.c. when given intramuscularly, was found to kill at 1/30,000 c.c., and not at 1/100,000 c.c. when given intravenously.

DISCUSSION.

The peculiar interest of the foregoing experiments is that a mixture of virus with its immune serum should show so close an analogy in its behaviour with that of toxin-antitoxin mixtures, in the case of which the occurrence of the dilution phenomenon has been recorded by a number of observers. Thus, working on mice with tetanus toxin-antitoxin mixtures, Behring and his co-workers found that a suitably prepared mixture of toxin and antitoxin became more toxic, up to a certain point, as the dose was diminished, so that the same volume of a 1 in 50 dilution, and even of a 1 in 500 dilution, of the mixture was more toxic than the original undiluted mixture. This question was dealt with somewhat fully by Behring (1899) in his 'Allgemeine Therapie der Infektionskrankheiten,' and again in 1904 after considerable further work on the subject.

Behring's first explanation of the phenomenon was that a chemical union takes place between the toxin and antitoxin, but that this union takes place much more slowly in dilute solutions. He believed that this was confirmed by the fact that dilute mixtures became non-toxic if kept for a sufficiently long time. Later, however, he was convinced that the latter result was simply due to the deterioration of the toxin on keeping.

His second explanation was that the phenomenon was due to a dissociation of the toxin-antitoxin compound at higher dilutions, and after much work on the subject with Romer, they believed that this hypothesis accounted for the facts.

On further study, however, he abandoned this view also, and in 1904 came to the conclusion "das wir mit einer Hypothese zu rechnen haben, die hinausläuft, das in vitro eine chemische Bindung zwischen Antitoxin und Gift entweder nur unvollständig oder gar nicht stattfindet, und dass unter

gewissen Umständen der antitoxische Entgiftungsprocess sich erst in vivo abspielt." This from Behring, as the result of some years' careful work on the subject, is of the greatest interest.

Madsen (1906) obtained the dilution phenomenon with mixtures of botulism toxin and antitoxin. He also found that hæmolytic experiments carried out with mixtures of saponin and cholesterol showed the phenomenon well. Madsen does not enter into the mechanism of the phenomenon and gives no suggestions as to its causation, but he notes that Biltz (1904) has described similar optima in inorganic precipitations and that possibly these might point to an explanation.

Otto and Sachs (1906) entirely confirmed Madsen's work. They found that if botulism toxin and antitoxin were mixed and the mixture then diluted and tested by subcutaneous injection into mice, it made practically no difference whether the dilution was made at once or not until three hours after mixing. Experiments were then made on rabbits, and it was found that toxin-antitoxin mixtures after 3 hours' contact were considerably more toxic for rabbits when given intravenously than when given subcutaneously, but that after 24 hours' contact the toxicity was approximately the same by either route. They believed that they were here dealing with the phenomenon observed by Morgenroth (1904), who found that diphtheria toxin and antitoxin took some time to combine, but that this could only be shown by intravenous injection, and that if the mixtures were injected subcutaneously the time of digestion had not the slightest influence. Morgenroth concluded from the results of his experiments that certain conditions are present in the subcutaneous tissue which accelerate the union of toxin and antitoxin—presumably the presence of something of the nature of a catalyser.

Otto and Sachs believed that the increased toxicity of dilutions of a toxin-antitoxin mixture can be explained by the presence of a catalyser; as, if the mixture when injected subcutaneously contains free toxin and antitoxin, the catalyser will presumably act more slowly in dilute solutions. In order to avoid the subcutaneous tissue Otto and Sachs tried intravenous injection, but found that here also the dilutions were definitely more toxic, although the toxin and antitoxin had been kept in contact for 3 hours. After 24 hours' contact between the toxin and antitoxin the dilution phenomenon was not observed.

Otto and Sachs believe that there are two phases in the union of toxin and antitoxin, viz.:

(*a*) a first stage in which the union is comparatively loose and during which dissociation can to a certain extent take place;

(*b*) a second stage of firmer union where dissociation is impossible or practically so;

and hold that the first stage is demonstrated by the dilution phenomenon and the second stage by the Danysz phenomenon—*i. e.* the increased toxicity of toxin-antitoxin mixtures on the fractional addition of the toxin.

Otto and Sachs were also able to demonstrate the dilution phenomenon in hæmolytic experiments with arachnolysin and its antiserum. On allowing the mixtures to stand for varying times before dilution they found that the dilution phenomenon was very striking after 1 hour and much less so after 24 hours, although it was still detectable after 48 and 72 hours.

The phenomenon has also been described in the case of diphtheria toxin-antitoxin mixtures, and Glenny (1925) in this connection states that "it is possible to produce a mixture of toxin and antitoxin causing no reaction when injected undiluted into the skin of an animal; when diluted 1/100 of the dose will produce a marked intracutaneous reaction. Similar mixtures cause no œdema when injected subcutaneously into guinea-pigs, but after absorption from the site of injection sufficient toxin becomes free to kill the animal in 5 days. Most ordinary mixtures of toxin and antitoxin in which the toxin has been sufficiently neutralized to prevent the appearance of any œdema when injected subcutaneously into an animal will cause late paralysis."

Dr. P. Hartley (personal communication *re* unpublished work) has observed the dilution phenomenon in the case of tetanus toxin-antitoxin mixtures, and has also found that the degree of such dissociation varies with different antitoxins.

In view of our lack of knowledge as to the nature of the ultramicroscopic viruses and of the mechanism by which protection against these is effected by immune sera, any attempted explanation of the dilution phenomenon in the case of a virus would obviously be premature until we are in possession of further information as to the relation of the virus to its immune serum.

SUMMARY.

1. A mixture of fowl plague virus with the corresponding immune serum, so prepared as to be just non-virulent when injected intramuscularly into a fowl, is rendered virulent by simple dilution with saline.

2. This dilution phenomenon takes place even after the undiluted mixture has been kept at 37° C. for 4 hours, showing that the action of the immune serum is not that of destroying the virus *in vitro*, but that the virus and immune bodies can exist side by side in the mixture without destruction of the former.

3. Similarly a mixture of the virus with just enough immune serum to render the mixture harmless to fowls when injected intramuscularly is found on intravenous injection to give rise to acute fowl plague, although the fowl is apparently not more susceptible to intravenous than to intramuscular injection of the virus.

4. This behaviour of mixtures of the virus with its immune serum resembles the behaviour of toxin-antitoxin mixtures observed in the case of tetanus, diphtheria and certain other toxins.

5. The dilution phenomenon is of practical importance in the titration of immune sera against the corresponding viruses, as the degree of dilution of the infected mixture may, within certain limits, influence the result of the injection.

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AN APPARATUS FOR MIXING CULTURES OR OTHER FLUIDS.

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THE forms of apparatus in common use in bacteriological technique for mixing fluids have certain disadvantages. They are usually cumbersome, and in most the motive power is supplied by an electric motor, which has obvious disadvantages when it is desired to continue the mixing process for many hours or days, especially in the hot room.

The apparatus to be described has no moving parts, requires practically no attention and can be improvised rapidly in any laboratory. It enables small or large volumes of fluid to be mixed either on the bench or in an ordinary small incubator, and allows the mixing process to be accurately controlled, and to be independent of the temperature, volume or viscosity (within limits) of the fluids to be mixed.

The fluid to be mixed is alternately sucked up and blown down a tube, the impetus being supplied by alternate emptying by siphonage and filling from the tap of a closed vessel.

ARRANGEMENT OF APPARATUS.

The apparatus is set up as in Fig. 1. It is represented as mixing four cultures through the tubes H, K, X, Y. The ends of the three tubes inside the bottle should occupy approximately the positions shown. The siphon-tube should be of wide bore in order to siphon quickly, and its inner end should be expanded as by attaching a small funnel, in order to break the column of water easily. The relatively narrow tube, E, has a distributor at its end, which connects it with the water releases (+ and −) and with the mixing-tubes to be described.

The following measurements should be noted:

The vertical height, C D, determines the greatest negative pressure which can be attained. The short limb, C, must be longer than the equivalent of the greatest positive pressure to be employed and shorter than E, as otherwise the water will be blown into the releases. The excursion of the water level between C and E determines the greatest bulk of fluid which can be mixed per cycle.

WORKING.

The apparatus is worked as follows:

Turn on the water so that the rate of inflow is about half the rate of siphonage by the siphon, previously determined. When the water-level reaches C (Fig. 1), the pressure in the system will rise until air is blown out of the tube F (positive release). The degree of pressure can therefore be adjusted by vertical movement of the tube F. At the same time the fluid inside the tubes H, K, X, Y will be depressed and the height of F is adjusted so that air

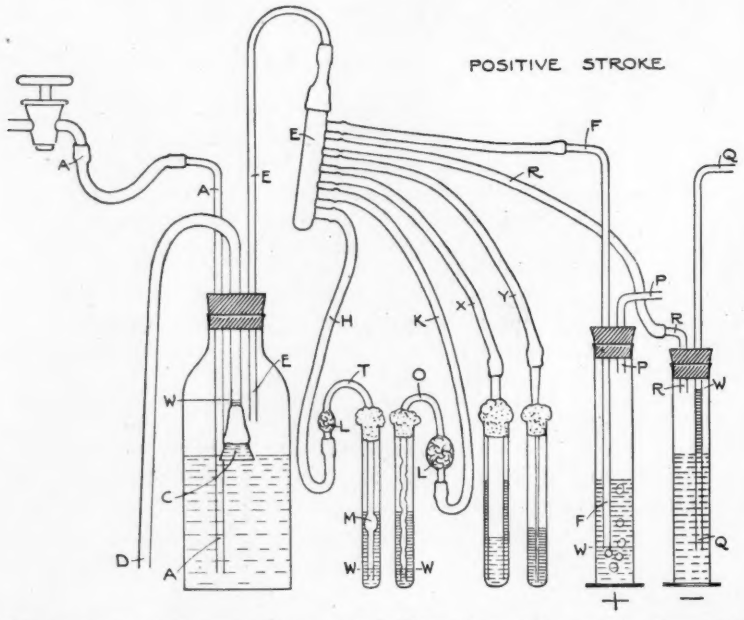

FIG. 1.—*Positive stroke commencing.*—Air is bubbling through the positive release and the water in the mixing-tubes is forced down to the same extent. At the same time the water in the short limb of the siphon C and in the negative release is raised. The water level in the large container is rising. The tubes X and Y, which are not described in the text, demonstrate that the fluid is depressed to the same extent whatever the bore of the mixing tube.

is not blown through the cultures. When the water in the jar reaches E no more air will be blown out, and the level will rise in C until the siphon is established (positive stroke). The rapid emptying of the bottle will cause a sharp fall in pressure in E, so that the fluids in F, H, K, X, Y will be sucked up. The pressure will continue to decrease until the minus release comes into action, and air is sucked in through Q. Tube Q (negative release) is also vertically adjustable to regulate the negative pressure and prevent fluid being sucked up into the bulbs L (negative stroke—Fig. 2).

During this phase water from the tap, A, has been running in constantly, but was so adjusted that the siphon would overtake it easily. When the siphon breaks the water-level commences to rise at once, and the whole cycle is repeated. The amount of water used is half the volume of fluid mixed per cycle and in practice is quite small.

A mixing-tube connected to the tube E, as at X, Y, H, K, would have communicated to it the same variations of pressure as were determined by the releases.

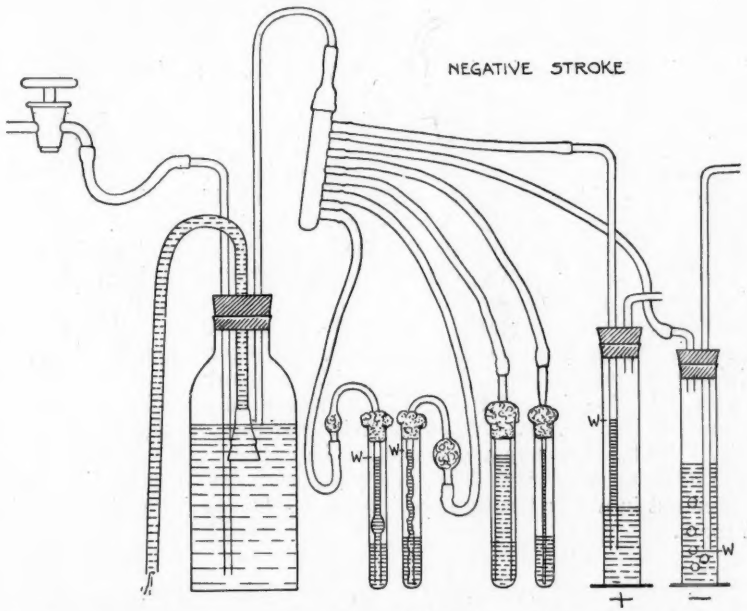

FIG. 2.—*Negative stroke commencing.*—The negative release is now sucking in air, and the broth in the mixing-tubes is drawn up to the same extent. At the same time the water in the positive release is drawn up and the siphon is working. The excursion of water in the large container is greater than is necessary to mix the small volume of fluid, but is so arranged to demonstrate the excursion of water more clearly. The water level in the large container is falling.

Mixing is thus performed by alternately sucking up and blowing down the fluid to be mixed. In the diagram the fluid is broth contained in test-tubes and the mixing-tubes are led in through plugs of sterile wool. Two forms of tube have been used. The first (T) consists of a plain glass tube of suitable bore, in which two small bulbs are blown (L and M); M is in such a position that the fluid within the tube falls below it during the positive stroke. Thus the fluid in the tube itself is constantly mixed as well as that in the test-tube. The bulb L is packed with sterile wool so that no dust or other contamination

from the apparatus can be blown into the culture. A more efficient but somewhat more complicated tube is also shown (o). It has the bulb L as in the other form, but the vertical part of the tube in which mixing takes place is indented in many places. These indentations are best made by sealing the ends of the tube while hot and then allowing the tube to collapse at the points desired by applying a fine flame. This form of tube is particularly useful where much aeration of a culture is desired.

Such mixing-tubes are easily sterilized by heat, and any number may be connected to the same apparatus by means of a distributor, such as Carrell's glass distributor used in the irrigation of wounds.

In order to mix a number of cultures in a standard type of incubator a tube may be led through the thermometer hole of the latter and connected to a distributor inside. To this distributor any number of mixing tubes may be connected.

In setting up the apparatus the mixing-tubes are arranged dipping into the various fluids to be mixed, say a series of cultures, and connected to a distributor. This is connected by a length of rubber-tubing—or preferably glass-tubing with rubber connections, as this is rigid and transmits variations of pressure more efficiently—from the main apparatus. The releases are then set at the required levels and the water turned on at a suitable speed. The level of the outlet-tube, E, is next adjusted so that in both the fixed positive and negative releases air bubbles through the water, showing that the desired pressures are being attained, *i. e.* that the volume excursion per cycle is sufficient. Once adjusted for a particular experiment the speed and degree of mixing remains constant indefinitely. The apparatus is applicable to fluids of considerable viscosity, such as blood, and to volumes ranging from 20 c.mm. to several hundred c.c.'s. Moreover fluids varying greatly in amount and viscosity and at different temperatures can be mixed equally at the same time.

It is a further advantage that the apparatus enables dynamic incubation to be carried out in a standard type of incubator, thus avoiding the necessity for a hot room.

APPLICATION TO ANAEROBIC CONDITIONS.

An adaptation of this apparatus for mixing anaerobic cultures has been devised. It consists of a disconnecting device by means of which the variations of air pressure from the mixing-machine previously described are transmitted by means of an inverted syphon to non-oxygen-containing gas derived from the anaerobe apparatus in connection with which it is to be used.

Very little adaptation of a standard McIntosh and Fildes jar is required. The two taps provided for the admission of hydrogen are replaced by a three-way and two-way piece respectively, each arm of which is provided with a tap. The diagram (Fig. 3) shows clearly the general lay-out of the apparatus. The fluid in the disconnecting device is pyro-soda, and a layer of liquid paraffin floats on the surface of this in the air-container (1) connected to the mixing apparatus. Liquid paraffin alone is not sufficient as an oxygen lock.

The apparatus is set up as follows: Liquid paraffin is run into the container 1, through the tap A to a depth of about $\frac{1}{2}$ in. The air in container 2 is now

replaced with coal-gas, which is allowed to run in at F and out at C while G, H and K are closed and E and D open, the lid of the apparatus being removed at this stage. A funnel is now attached to C and caustic soda solution is run in, the tap A being closed so that no fluid flows over the inverted syphon into 1 at this stage. A solution of pyrogallic acid is now run in until the container 2 is full. In this way little oxidation of the reducing solution takes place. The tap C is now closed.

One or more cultures are set up as has been described and as is shown diagrammatically in the illustration, and connected to the point G, the cultures standing in the anaerobe jar in the usual way.

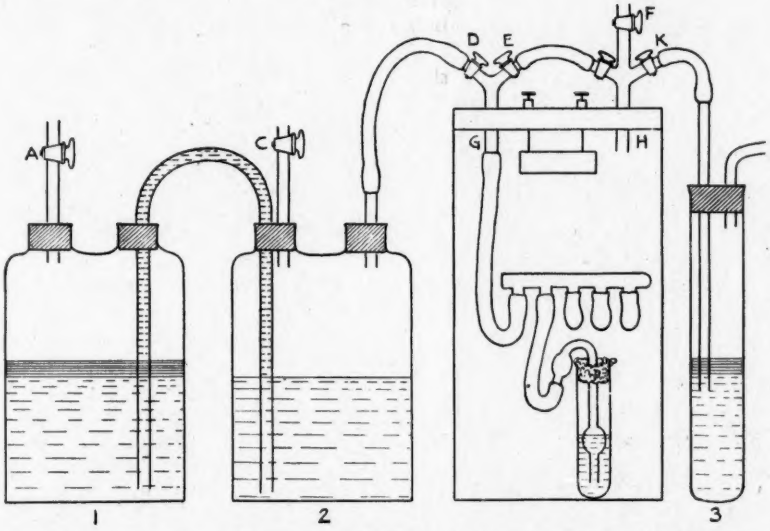

FIG. 3.—*Apparatus for use with anaerobic cultures.*—A is to be connected to the distributor E shown in Fig. 1.

The taps A, C and K are closed and the taps D, E and F opened. The hydrogen apparatus is connected to the point F, the lid of the jar put on and the electric current turned on so that oxygen replacement proceeds. Since the taps D and E are open replacement takes place not only in the jar, but also in the tubes of the apparatus, including the mixing-tubes themselves. Also the pressure in the mixing-tube is the same as that in the jar—E being open—and it is impossible for the cultures to be blown over by the hydrogen pressure.

When replacement is complete the tap K is opened slowly and gas allowed to bubble through the fluid in 3, which is pyro-soda on which a layer of liquid paraffin floats. In this way any oxygen in the tube is replaced. K is now closed and A is slowly opened. Gas from the apparatus which is under pressure enters through D and the level of the fluid in 2 falls, being blown over the inverted syphon into 1. When this is the same as that in 1, A is closed. F is

now closed, and the remaining positive pressure in the apparatus is released by opening K. A is then opened, and the levels in 1 and 2 are equalized by the inverted syphon.

All this apparatus is now placed in the incubator and the point A connected to the mixing device described in the earlier part of the paper. The tap E is now closed so that the mixing-tube is connected to the mixing apparatus and disconnected from the contents of the anaerobe jar.

If the mixing is now started the cultures will be mixed quite anaerobically. As the gas in the jar is heated it expands and this excess escapes at K, so that there is no interference with the mixing system.

When it is desired to take out the cultures the tap E is opened and the mixing stopped, the lid of the jar is removed and the mixing-tubes disconnected.

Dynamic incubation of bacteria is easily performed by means of this apparatus. Bacteria which prefer a plentiful supply of oxygen give a denser growth in a given time if grown in this way. Staphylococci and *B. pyocyaneus* give four times the usual density of culture (numerically) in 18 hours if grown dynamically and a culture of the latter organism is then bright green in colour. Anaerobic bacteria also give a denser growth in a given time when grown in this way. This may be due to the mixing of the broth, causing it to give up its contained oxygen more quickly, so that strict anaerobiosis within the medium is attained sooner. In phagocytosis experiments it has been found quite practicable to mix completely as little as 20 c.mm. of blood in a pipette stem. Dr. Alexander Fleming has also employed the apparatus to investigate the rate of agglutination of bacteria under different conditions of temperature.

The device can be used successfully in many ways, and may have a wide application in laboratory technique where thorough and easily controlled mixing of fluids is required.

EXPERIMENTS ON THE PURIFICATION AND CONCENTRATION OF SCARLET FEVER TOXIN.

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In recent years many attempts have been made to discover a trustworthy and convenient method for the standardization of scarlet fever antitoxin. The method which has been used up to the present, and which has recently received official recognition in the United States of America, depends upon the intracutaneous injection of mixtures of scarlet fever toxin and antitoxin into susceptible human beings, the result being determined by the presence or absence of characteristic skin reactions of a certain size. There are certain obvious limitations and disadvantages attending the use of such a method, and other methods have been sought for which, in their application, would obviate the use of human beings as biological indicators. One of the main difficulties in the solution of this aspect of the scarlet fever problem is due to the fact that while a certain proportion of human beings are susceptible to scarlet fever toxin, no other species of animal has yet been discovered which exhibits an equal degree of susceptibility. According to Wadsworth, Kirkbride and Wheeler (1926), goats are susceptible to scarlet fever toxin and give a recognizable skin reaction, and the use of these animals for the purpose of scarlet fever antitoxin standardization has been advocated. Other workers have not been so successful in the application of this method, which must still be regarded as on its trial. Parish and Okell (1927, 1928) have carried out an extensive series of experiments, the results of which hold out some promise that a method of standardization may be evolved in which the rabbit may be used as test animal. These workers have shown that whereas rabbits injected with living cultures of the scarlet fever streptococcus die of acute septicæmia within a few days, rabbits which are given an injection of scarlet fever antitoxin four to six hours before the culture is injected are protected against the acute phases of the septicæmia but develop later a subacute infection. The protected animals either survive, or live for longer periods than the unprotected controls. Parish and Okell also showed that scarlet fever antitoxin, and to a lesser degree certain other streptococcus antitoxins, protected animals against scarlet fever *toxin*. They found, however, that it was necessary to inject large quantities of toxin—20 to 40 c.c.—intravenously in order to be certain of producing a fatal issue, and the application of the test in this form is impracticable on account of the feeble potency of the toxin for rabbits and the irregularity of the results obtained.

It is generally agreed that the standardization of scarlet fever antitoxin

would be simplified if the titrations could be carried out on laboratory animals; and since no species of laboratory animal appears to be generally available which gives with regularity a definite specific skin reaction to the toxin, some general toxicity test, and preferably a lethal dose method, would, if available, solve some of the more urgent problems and eliminate some of the present difficulties. The fundamental requirement of such a development of the test is the employment of a much more potent toxin than has hitherto been available. The accuracy of tests of this nature is directly proportional to the number of fatal doses which can be included in the test dose of toxin used. From the results of the large amount of work which has been carried out on the preparation of scarlet fever toxin, it appeared unlikely that a suitable and sufficiently potent toxin could be prepared directly by ordinary cultural methods, as diphtheria and tetanus toxins are prepared. There remained the possibility of purifying and concentrating the crude and relatively weak toxins which are now available.

Several investigators have reported the results of experiments on the purification of scarlet fever toxin, although none of these appear to have tested their preparations on laboratory animals for toxicity. Huntoon (1923) added ammonium sulphate to 60% saturation, and 1% acetic acid, to the filtrates from 7-day growths on blood broth of streptococci isolated from cases of scarlet fever. The voluminous precipitate so obtained was filtered off and more ammonium sulphate added until the saturation reached 70 to 75%. A second precipitate was thus obtained which contained the toxic substance. Huntoon found that his purified product contained "so little nitrogen as to be within the realm of experimental error," and concludes from other experiments that the toxin is a protein substance inactivated by trypsin and by heat. The toxin was neutralized by serum from convalescent scarlet fever cases, but not by normal horse-serum nor by the serum of horses immunized by the injection of emulsions of the scarlet fever streptococcus. Henry and Lewis (1925) effected a considerable purification of scarlet fever toxin by means of alcohol precipitation. Mackie and McLachlin (1926) found that when 5-day growths of the scarlet fever streptococcus on rabbits' blood broth were heated to 56° C. for one hour, an abundant brown precipitate was formed which contained no active material. The filtrate, containing the toxin, was further purified by precipitation with 6 volumes of alcohol. Liprowska, Sierakowski and Sparrow (1927) subjected scarlet fever toxin to ultra-filtration through collodion membranes of varying permeability, and found that certain types of membranes retained the whole of the toxin while permitting the passage of about 80% of the nitrogenous constituents of the crude toxin.

Pulvertaft (1928) has recently succeeded in preparing scarlet fever toxins of much greater potency than have hitherto been obtained. His work has been in progress during the same period as my own. By private communication each of us has become acquainted with the other's results, reached by an essentially different method. We have arranged to publish them simultaneously, and his paper will be found on p. 276. Pulvertaft's toxins were lethal for rabbits in doses varying from 2·5 c.c. to 10 c.c., and he has found that the toxins can be purified by precipitation with alcohol, whereby 90% of the nitrogenous constituents of the crude toxin are removed, and concentrated

preparations obtained which are lethal for rabbits in doses as small as 0·1 c.c. Complete protection is afforded against the toxin by streptococcal antisera, but not by other sera.

The experiments described below were carried out in order to discover whether any of the methods which have been used in this Laboratory in recent years for the purification and concentration of antigens could be applied to scarlet fever toxin. The method which has yielded the most promising results is that described by Walpole (1915), and first used by Glenny and Walpole (1915) for the purification and concentration of diphtheria toxin. Briefly stated, this method consists of (i) dialysis of the crude toxin, saturated with toluol, under pressure through collodion membranes against distilled water, also saturated with toluol. (ii) Precipitation of the active substance from the dialysed toxin. (iii) Separation of the precipitated toxin by centrifuging; and (iv) re-solution of the precipitate in a greatly reduced volume of solvent. This method, when applied to scarlet fever toxin in two large-scale experiments, has yielded a final product containing relatively small amounts of nitrogen, and fatal for rabbits in doses of 0·5 c.c. and 0·2 c.c. respectively. The concentrated toxin so obtained is heat-labile, and is neutralized by a specific antitoxin, but not by normal serum.

EXPERIMENTAL.

1. *The Scarlet Fever Toxin Used.*

Two large batches, Nos. 188 and 195, of scarlet fever toxin were kindly supplied by Dr. R. A. O'Brien of the Wellcome Physiological Research Laboratories. The toxins were prepared by growing the Dochez strain of *Streptococcus scarlatinæ* on digest broth for 18 hours, filtration through pulp filters, adjustment of the reaction to pH 7·6, and finally, filtration through Berkefeld candles. Each toxin gave a good skin reaction at a dilution of 1 in 1000.

2. *Preliminary Experiments.*

350 c.c. quantities were dialyzed under pressure in a single large collodion bag against distilled water, the dialysis being continued for three days. The final product was highly toxic for rabbits, and the toxic properties were neutralized by a specific antitoxin, but not by normal serum. These preliminary experiments suggested that Walpole's method could be applied to scarlet fever toxin, and large-scale experiments were accordingly undertaken.

3. *The First Large-scale Experiment.*

A pressure dialysis apparatus consisting of three collodion bags in series was set up in the manner described by Walpole (*Biochemical Journal*, 1915, vol. ix, fig. 3, p. 290), the capacity of each bag being about 350 c.c. Three litres of scarlet fever toxin No. 188 were placed in the apparatus, and the dialysis conducted in the way described by Walpole (1915), and Glenny and Walpole (1915). The dialysis was carried out under a pressure of 10 to 15 cm. of mercury. It may be mentioned that during this stage of the process from 70 to 75% of the nitrogen present in the original toxin is

removed—a good deal more than has been observed with other bacterial toxins studied by this method.

The next stage of the process consists in the precipitation of the active substance from the dialyzed toxin. This has proved to be troublesome; and as other workers may meet with similar difficulties, the following details are given, since such success as has attended these experiments has been obtained by the adoption of a somewhat empirical technique. 10 c.c. quantities of the dialyzed toxin were placed in a series of tubes, and gradually increasing quantities of N. acetic acid (0·1 c.c. to 1 c.c.) added, and after thorough mixing, the tubes were maintained at room temperature and inspected for the appearance of a precipitate. An opalescence, increasing with increasing concentration of acetic acid, was first observed, and after about half an hour a very fine precipitate appeared, usually in the tube containing 0·5 c.c. of N. acetic acid, and later in other tubes on either side in the series. On standing for longer times precipitation was more obvious, and occurred in all, or nearly all the tubes. For the purpose of these experiments it was decided to adopt as "indicating tube" the lowest concentration of acid which caused precipitation in about half an hour. Other experiments showed that precipitation occurred in about half an hour when the pH of the dialyzed toxin was made 4 to 4·2. The whole of the dialyzed toxin was then precipitated with the requisite amount of N. acetic acid, and after standing for about half an hour the precipitate—extremely small in amount in all the experiments—was separated by centrifuging. The sediment was first emulsified in normal salt solution, and then decinormal sodium hydrate was added, drop by drop, until solution was complete. This occurred at about the neutral point, and the reaction was made pH = 7·4 by the further addition of decinormal alkali. The final solution, which constitutes the concentrated toxin, is a pale greenish-yellow or greenish-brown, slightly opalescent fluid, free, or nearly so, from visible particles.

(i) *The Degree of Concentration Attained.*

The following estimations were carried out on the original and concentrated scarlet fever toxin No. 188:

TABLE I.

	Original toxin No. 188.	Concentrated toxin No. 188.
Volume	3000 c.c.	150 c.c.
Total nitrogen per c.c.	4·91 mgm.	0·73 mgm.
Minimum lethal dose	? 10·0 c.c.	0·5 c.c.

That is, in this experiment over 99% of the nitrogen occurring in the original toxin was removed and the volume was reduced to one-twentieth.

On account of the difficulty of estimating accurately the minimum lethal dose of the original toxin it is not easy to determine the degree of concentration attained in respect of toxin units. Three rabbits which received 10 c.c. of original toxin intravenously all died—one in 18 hours, one in 3½ days and one in 4 days; one rabbit which received 20 c.c., and one which received 15 c.c., died in 18 and 24 hours respectively, while one which received 12·5 c.c.

was ill for 4 days, but survived. Two rabbits which received 7·5 c.c. both lived. Assuming that the minimum lethal dose of the original toxin is 10 c.c., and that of the concentrated toxin is 0·5 c.c. (see Table II), then a twenty-fold concentration in respect of volume, and more than a hundred-fold concentration in respect of nitrogen, has been effected with no significant loss of toxin.

(ii) *The Toxicity for Rabbits of the Concentrated Scarlet Fever Toxin No. 188.*

The results of these tests are shown in Table II. The doses given were injected intravenously.

TABLE II.

Dose of concentrated toxin No. 188.		Number of rabbits injected.	Result.
Volume, c.c.	Nitrogen, mgm.		
5·0	3·65	1	Died within 18 hours.
2·0	1·46	1	,, 18 ,,
1·0	0·73	9	All died within 18 hours.
0·75	0·55	10	9 died within 18 hours; 1 died in 30 hours.
0·50	0·36	10	6 died within 18 hours; 2 died in 3½ days; 2 lived.
0·25	0·18	1	Lived.
0·10	0·07	1	,,

The minimum lethal dose of the preparation is rather more than 0·5 c.c. and less than 0·75 c.c., and contains from 0·36 mgm. to 0·55 mgm. of nitrogen.

Symptoms and post-mortem findings.—After injection of a fatal dose of the concentrated toxin there is a latent period varying from 1½ to 6 hours during which the animal appears to be perfectly well. An increase in the respiration rate is then observed, and the animal lies on its side and shows incontinence of fæces. Diarrhœa then ensues and may become very intense. Death occurs usually in 14 to 18 hours. A certain number of animals died in 3 to 6 hours after injection, and it was noticed that the animals which died quickly were always females, and nearly always pregnant.

The most striking post-mortem features are shown in female rabbits, in which the mammary glands show patches of deep congestion around the nipples, and in pregnant animals the whole of the tissue of these glands may be involved. The uterus also shows congestion, which, in pregnant animals, is very pronounced. The stomach shows hæmorrhages, particularly in those animals which survive for 18 to 24 hours. The heart-blood remains fluid for several hours after death, and if the dose given is large, may show some lysis. The intestines are distended with gas and the splanchnic venous system is engorged.

No special importance is attached to the symptoms following injection, since these are such as have been described as being produced by the intravenous injection of other substances, and particularly other bacterial toxins.

On the other hand, the post-mortem findings suggest that the scarlet fever toxin may have a special affinity for the cells of the mammary gland and the uterus. It is, further, of possible significance that early death, when it occurs, does so in pregnant females. In this connection it is interesting to note that one animal, in an advanced stage of pregnancy, which received a sub-lethal dose of the concentrated toxin, exhibited the usual symptoms in a mild form and during the night following the injection aborted. The animal recovered. These observations suggested that possibly a specific action of the toxin might be demonstrated on isolated rabbits' uterus. One experiment of this kind was carried out by my colleague, Dr. J. H. Gaddum, but no such immediate action could be demonstrated.

An experiment was carried out to determine whether toxic substances could be isolated from uninoculated digest broth: 350 c.c. of the medium were treated by Walpole's method, and an amount of material, representing the concentrated product from 120 c.c. of broth, was injected intravenously into a female rabbit. The animal remained perfectly well and showed none of the symptoms noted above. It was killed 36 hours after injection, and the mammary glands, uterus and stomach appeared quite normal.

(iii) *The Effect of Scarlet Fever Antitoxin on Concentrated Scarlet Fever Toxin No. 188.*

These experiments were carried out in the following way: 1 c.c. of concentrated scarlet fever toxin (*i.e.* slightly less than two minimum fatal doses) was added to varying quantities of antitoxin, and, after thorough mixing, the mixtures were incubated at 37°C. for 1 hour. One or more rabbits were included in each series of tests as controls; these animals were given 0·75 c.c. or 1 c.c. of toxin alone, the toxin doses being first diluted to 2 c.c. with normal saline and incubated at 37°C. for 1 hour. The doses were injected intravenously. No precipitation occurred in the mixture of toxin and antitoxin.

Five different samples of scarlet fever antitoxin were examined. All the samples had been prepared for use in the treatment and prophylaxis of scarlet fever; two had been manufactured in this country, two in America and one in Germany. The tests were carried out on different days and the results are collected in Table III.

The tests are not very extensive, but as far as they go they reveal several points of interest. The ten control animals all died acutely. The titration of four of the samples of antitoxin viz. P, W, S and D, gave perfectly regular results. If tests carried out in this rather crude way (*i.e.* in which the test dose of toxin contains such a small multiple of the fatal dose and in which the number of animals used is small) can be given any quantitative significance, it would appear that the relative values of the four antitoxins are in the order P>W>S and D, the doses of antitoxin required to protect the life of a rabbit against the same test dose of toxin being 0·25, 0·5, 1 and 1 c.c. respectively. The results of the test of the antitoxin B.S. are not so regular. The six animals used in this test remained well for 2 days. Of the four which received the smaller doses of antitoxin, three became ill on the third day and displayed

much the same symptoms as the control, unprotected animals exhibited some hours after injection. The animals became paralysed and two subsequently died.

TABLE III.

Number of scarlet fever antitoxin.	Mixture injected. Concentrated toxin No. 188. c.c.	Mixture injected. Antitoxin. c.c.	Number of rabbits used.	Result.
P.	1·0	1·5	1	Lived; no symptoms.
	1·0	1·0	1	„ „
	1·0	0·5	1	„ „
	1·0	0·25	1	„ „
	1·0	0·125	1	Died within 18 hours.
W.	1·0	1·0	2	Both lived; no symptoms.
	1·0	0·5	2	„ „
	1·0	0·25	3	2 died in 2½ days; 1 died in 4½ days.
S.	1·0	2·0	1	Lived; no symptoms.
	1·0	1·0	1	„ „
	1·0	0·5	1	Died in 4 days.
	1·0	0·25	1	„ 1½ „
D.	1·0	1·0	2	Lived; no symptoms.
	1·0	0·5	2	1 died in 2½ days; 1 died in 1½ days.
B.S.	1·0	1·5	1	Lived.
	1·0	1·0	1	„
	1·0	0·5	2	No symptoms until 3rd day, then both became paralysed; 1 recovered and 1 died.
	1·0	0·25	2	1 lived; no symptoms; 1 showed no symptoms until 3rd day, then became paralysed and died on 4th day.
Controls	1·0	*Nil*	3	All died within 18 hours.
	0·75	„	7	„ „ 18 „

(iv) *The Action of Other Anti-Streptococcic Sera, and of Normal Horse-serum, on Concentrated Scarlet Fever Toxin No. 188.*

This series of experiments includes:

(*a*) Two samples of puerperal streptococcus antitoxin.

(*b*) Three samples of anti-streptococcus serum known to have been prepared by the injection of emulsions of streptococci, the strains being other than *Streptococcus scarlatinæ*.

(*c*) Normal horse-serum.

The experiments were carried out in a similar way to those described in the preceding paragraph. The results are collected in Table IV:

TABLE IV.

Number and kind of serum.	Mixture injected. Concentrated toxin No. 188. c.c.	Serum. c.c.	Number of rabbits used.	Result.
Puerperal streptococcus antitoxin, L.	1·0	2·0	1	Lived; no symptoms.
	1·0	1·0	1	„ „
	1·0	0·5	1	„ „
	1·0	0·25	1	„ „
	1·0	0·125	1	Died in 3½ days.
Puerperal streptococcus antitoxin, B.	1·0	1·0	2	1 lived; one died within 18 hours.
	1·0	0·5	2	Ditto.
Anti-streptococcic serum (anti-bacterial, non-scarlatinal) Dp.	1·0	2·0	1	Died in 3 hours.
E.	1·0	2.0	1	„ within 18 hours.
A.	1.0	2.0	1	„ „ 18 „
Normal horse-serum	1·0	3·0	1	Died in 3 hours.
	1·0	2·0	1	„ within 18 hours.
	1·0	1·0	2	„ „ 18 „
Controls	1·0	*Nil*	1	Died within 18 hours.
	0·75	„	6	All died within 18 hours.

Normal horse-serum and antistreptococcus serum prepared against emulsions of organisms as distinct from toxin (the strains used being other than the *Streptococcus scarlatinæ* strain) exhibit no neutralizing properties when tested against the concentrated scarlet fever toxin. On the other hand, one sample of puerperal streptococcus antitoxin (viz. L, a concentrated globulin preparation) was found to be as effective as scarlet fever antitoxin in neutralizing the concentrated toxin. The other sample of puerperal streptococcus antitoxin is apparently of lower potency, but nevertheless shows some neutralizing properties. The results of these few experiments support the view advanced by Okell and Parish (1928) that the commonly occurring pathogenic hæmolytic streptococci produce the same toxin.

(v) *The Effect of Desiccation on Concentrated Scarlet Fever Toxin No. 188.*

It has been found that toxins, when concentrated by Walpole's method, are unstable in solution and are rapidly destroyed by carbolic acid. In order to preserve the activity of the preparations obtained in these experiments the solution of concentrated toxin finally obtained was distributed in 2·5 c.c. quantities in sterile phials and exposed to the dehydrating action, first of concentrated sulphuric acid and then of phosphorus pentoxide. The desiccated product dissolved easily, and yielded a solution indistinguishable, as regards appearance and toxicity, from the original concentrated toxin. The toxicity remained unaltered four months afterwards when the contents of the last remaining phial were redissolved and tested.

(vi) *The Effect of Heat on the Activity of Concentrated Scarlet Fever Toxin No. 188.*

The effect of heating the concentrated toxin solution in sealed phials for 1 hour at 100° C. and at 56° C. was investigated. One animal which received 1 c.c. of toxin heated to 100° C. for 1 hour showed no symptoms and lived. One animal which received 1 c.c. of toxin which had been heated to 56° C. for 1 hour remained well for 3 days, but died on the fourth day, and one which received 0·75 c.c. of the same heated preparation lived. Since 0·5 c.c. is almost invariably fatal for rabbits, these few results suggest that the toxin is weakened by heating to 56° C. for 1 hour and destroyed, or very greatly weakened, by heating to 100° C. for 1 hour.

4. *The Second Large-scale Experiment.*

15·6 litres of a somewhat stronger scarlet fever toxin, No. 195, were used in this experiment. The pressure dialysis was conducted in an apparatus consisting of eight 350 c.c. collodion bags in series. The dialysed toxin, when brought to a reaction of pH = 4, yielded a very minute amount of solid—much smaller than in the first large-scale experiment. The volume of the final concentrated solution was made up to 390 c.c. A small amount of this solution was retained for immediate experiment and the rest was dried in the manner already described.

The data regarding this second sample of concentrated toxin are submitted in note and tabular form below.

(i) *The Degree of Concentration Attained.*

TABLE V.

	Original toxin No. 195.	Concentrated toxin No. 195.
Volume	15,600 c.c.	390 c.c.
Total nitrogen per c.c. . .	3·86 mgm.	0·84 mgm.
Minimum lethal dose . .	? 7·5 c.c.	0·2 c.c.

As in the first large-scale experiment, over 99% of the nitrogen occurring in the original toxin was removed during the processes of preparation. The volume in this case was reduced to one-fortieth. The lethal dose of the original toxin was estimated to be about 7·5 c.c. (doses of 20 c.c., 10 c.c. and 7·5 c.c. proved fatal; animals receiving doses of 5 c.c. survived), and if the lethal dose of the concentrated toxin be taken as 0·2 c.c. (see Table VI), then a 40-fold concentration in respect of volume and a 170-fold concentration in respect of nitrogen has been effected with little loss of toxin.

(ii) *The Toxicity for Rabbits of Concentrated Scarlet Fever Toxin No. 195.*

The minimum lethal dose of the concentrated toxin is rather more than 0·2 c.c. but less than 0·25 c.c., and contains from 0·17 mgm. to 0·21 mgm. of nitrogen.

TABLE VI.

Dose of concentrated toxin No. 195.		Number of rabbits tested.	Result.
Volume, c.c.	Nitrogen, mgm.		
1·0	0·84	2	Both died within 18 hours.
0·75	0·63	2	„ „ 18 „
0·50	0·42	12	All died within 18 hours.
0·30	0·25	8	7 died within 18 hours; 1 died in 5 days.
0·25	0·21	4	All died within 18 hours.
0·20	0·17	11	5 died within 18 hours; 1 died in 3 days; 1 died in 5 days; 4 lived.
0·15	0·13	12	5 died within 18 hours; 2 died in 1½ days; 5 lived.
0·10	0·08	3	All lived.
0·05	0·04	1	Lived.

(iii) *The Effect of Scarlet Fever Antitoxin, and of Normal Horse-serum, on Concentrated Scarlet Fever Toxin No. 195.*

These experiments were carried out in the following way: The dose of scarlet fever antitoxin, or of the normal horse-serum, was injected intravenously into one ear of the rabbit, and about 5 hours afterwards the dose of concentrated scarlet fever toxin was injected into the other ear. Control animals, receiving the dose of toxin *alone* shown in the table, were included in all experiments. The experiments were carried out on different days and the results are collected in Table VII.

Thus, of the fifteen rabbits which received 1 c.c. or more of the specific antitoxin 5 hours before the toxin, fourteen survived the subsequent injection of four to five lethal doses. 0·5 c.c. of the antitoxin protected two rabbits out of four against the same dose of toxin, while four animals which received smaller doses of antitoxin all died. It is interesting to compare these results with those given in Table III (under P.), having regard to the fact that in the earlier experiment the toxin and antitoxin were mixed and then injected, whereas in this experiment the antitoxin was given 5 hours before the toxin.

(iv) *The Effect of Heat on Concentrated Scarlet Fever Toxin No. 195.*

Two rabbits were injected with 1 c.c. and 0·5 c.c. respectively of concentrated scarlet fever toxin solution No. 195, which had been heated in a sealed capsule for 1 hour at 100° C. Neither animal showed any symptoms of illness and both lived.

Three rabbits were injected with concentrated scarlet fever toxin solution No. 195, which had been heated to 56° C. for 1 hour. One animal which received 0·65 c.c. of the heated toxin died acutely; one which received 0·5 c.c. and one which received 0·3 c.c. both lived and showed no symptoms.

Since the minimum lethal dose of the concentrated toxin is about 0·2 c.c.,

these experiments show that heating to 100° C. for 1 hour practically destroys the toxin, while heating to 56° C. for 1 hour weakens the toxin considerably.

TABLE VII.

Serum.	Injected with— Serum.	Concentrated toxin No. 195.	Number of rabbits tested.	Result.
Scarlet fever antitoxin, P.	10·0	1·0	1	Lived; no symptoms.
	5·0	1·0	4	All lived; no symptoms.
	2·5	1·0	6	5 lived; 1 died within 18 hours.
	1·5	1·0	2	Both lived; no symptoms.
	1·0	1·0	2	Ditto.
	0·5	1·0	4	2 lived; 1 died within 18 hours; 1 died in 4 days.
	0·25	1·0	2	1 died in 4 days; 1 died in 5 days.
	0·20	1·0	1	Died within 18 hours.
	0·10	1·0	1	„ 18 „
Normal horse-serum	5·0	0·5	3	All died within 18 hours.
	2·0	0·3	1	Died within 18 hours.
Controls	*Nil*	0·50	4	All died within 18 hours.
	„	0·30	4	3 died within 18 hours; 1 died in 5 days.
	„	0·25	2	Both died within 18 hours.
	„	0·20	7	3 died within 18 hours; 1 died in 3 days; 1 died in 5 days; 2 lived.
	„	0·15	7	4 died within 18 hours; 3 lived.
	„	0·10	2	Both lived.

(v) *The Effect of Desiccation of Concentrated Scarlet Fever Toxin No. 195.*

Experiments similar to those described under Paragraph 3 (v) were carried out with this preparation, and it was demonstrated that the concentrated toxin could be reduced to the dry condition without loss of activity. The major portion of this concentrated toxin No. 195, after having been reduced to dryness by exposure to the dehydrating action of concentrated sulphuric acid, was lost on account of the collapse of the desiccator in which it was being dried, and accordingly further experiments which had been projected could not be performed.

5. *The Action of Concentrated Scarlet Fever Toxin on Other Species of Animals.*

On account of the loss of material, due to the accident referred to above, it has not been possible to complete the experiments begun on this subject. The results of the few experiments so far carried out show that mice, rats and guinea-pigs are much less susceptible to the concentrated scarlet fever toxin than rabbits. Mice and young white rats died after the intravenous injection of 0·2 c.c. to 0·5 c.c. quantities of concentrated scarlet fever toxin No. 188, but survived the injection of smaller quantities. Guinea-pigs became very ill 2 hours after the intravenous injection of 0·5 c.c. to 1 c.c. quantities of the same preparation, and remained dull, lethargic and without appetite for about 2 days, but ultimately recovered.

Skin tests were carried out on guinea-pigs, rabbits and goats. Guinea-pigs showed no reaction, while rabbits gave a small reaction to the concentrated preparation diluted five times with normal saline. The concentrated scarlet fever toxin No. 195 was tested on the skin of two goats. A large, diffuse and rather pale reaction was produced by the injection of 0·02 c.c. of the concentrated preparation and a pale but recognizable reaction was produced by the injection of 0·004 c.c. Doubtful reactions were produced by smaller doses. All these reactions faded quickly. The two goats used were both young animals, under 6 months old, and this may account, in part, for their relative insusceptibility, since Wadsworth, Kirkbride and Wheeler (1926) found that young animals are less susceptible than goats from 2 to 4 years old. There is also the possibility that goats bred in different countries may vary in their susceptibility to scarlet fever toxin.

6. *The Action of Concentrated Scarlet Fever Toxin on Man.*

It appeared to be of some interest and importance to determine whether the active principle, present in culture filtrates of *Streptococcus scarlatinæ*, which causes a positive reaction in the skin of susceptible human beings, had been concentrated by the method of purification adopted.

The experiments carried out to investigate this point were performed by Dr. A. Joe and Dr. R. Swyer of the North Western Hospital, and it is a pleasure to record my thanks to them for their kind co-operation.

One c.c. of concentrated scarlet fever toxin No. 195 was diluted to 100 c.c. in the borax-boric acid buffer solution described by Glenny, Pope and Waddington (1928), and filtered through a Berkefeld candle. The sterile filtrate was further diluted with the same buffer solution to make dilutions of 1 : 20,000 and 1 : 40,000, and 0·2 c.c. quantities of these two dilutions were injected intracutaneously into the skin of a susceptible adult (P. H.). Large typical reactions were produced measuring 35 mm. by 35 mm. and 25 mm. by 30 mm. respectively. The reactions persisted for 48 hours with little change in intensity and then became paler and later showed pigmentation. This subject was also injected with 0·2 c.c. of a 1 in 1000 dilution of ordinary scarlet fever toxin heated to 100° C. for 2 hours, and with 0·2 c.c. of a *1 in 1000* dilution of the concentrated scarlet fever toxin which had also been heated to 100° C. for 2 hours. A negative reaction was obtained in each case.

A week later further tests were carried out on fifteen other persons. Each was injected intracutaneously with the following quantities of ordinary and concentrated scarlet fever toxin:

(i) 0·2 c.c. of a 1 in 1000 dilution of ordinary scarlet fever toxin.
(ii) 0·2 c.c. of a 1 in 20,000 dilution of ordinary scarlet fever toxin.
(iii) 0·2 c.c of a 1 in 20,000 dilution of concentrated scarlet fever toxin.

The 20,000-fold dilution of the concentrated toxin was used rather than the 40,000-fold dilution, because the dilutions had been made for a week before being tested, and it was thought that some deterioration in value might have occurred. The reactions were measured 22 hours after injection. The results are shown in Table VIII:

TABLE VIII.

No. of case.	Reaction produced by intracutaneous injection of 0·2 c.c. of— Ordinary scarlet fever toxin, 1 : 1000.	Ordinary scarlet fever toxin, 1 : 20,000.	Concentrated scarlet fever toxin, 1 : 20,000.
1	...	...	...
2	20 mm. × 17 mm.	10 mm. × 10 mm.	22 mm. × 22 mm.
3	27 „ × 25 „	10 „ × 10 „	32 „ × 25 „
4	24 „ × 24 „	10 „ × 11 „	20 „ × 19 „
5	46 „ × 23 „	21 „ × 18 „	35 „ × 25 „
6	20 „ × 19 „	...	19 „ × 17 „
7	26 „ × 18 „	...	23 „ × 20 „
8	...	...	...
9	...	...	...
10	24 „ × 19 „	...	26 „ × 21 „
11	23 „ × 19 „	...	20 „ × 17 „
12	...	...	...
13	...	...	25 „ × 21 „
14	...	...	...
15	25 „ × 23 „	12 „ × 7 „	25 „ × 23 „

... = no reaction.

The fifteen cases may be divided into three categories, and the results of the tests with ordinary and concentrated toxins considered in respect of each.

(i) Five cases (Nos. 1, 8, 9, 12 and 14) showed no reaction to either toxin.

(ii) Four cases (Nos. 6, 7, 10 and 11) reacted positively to 1 : 1000 ordinary toxin, and reacted positively, and to an equal degree, to 1 : 20,000 concentrated toxin; these four cases reacted negatively to 1 : 20,000 ordinary toxin.

(iii) Five cases (Nos. 2, 3, 4, 5 and 15) reacted positively to 1 : 1000 of the ordinary toxin, and reacted positively, and to an equal degree, to

1 : 20,000 of the concentrated toxin. These five persons also reacted positively to 1 : 20,000 of the ordinary toxin, although in four of these cases the reaction is very small.

One case only—No. 13—reacted in an anomalous way, in so far as a positive reaction was given by the concentrated toxin and a negative reaction by the ordinary toxin. Dr. Joe and Dr. Swyer considered that, on the whole, the reaction to the concentrated toxin was somewhat greater than the reaction to the ordinary toxin, and if the dilution of the concentrated toxin should in fact contain a little more active substance than the dilution of ordinary toxin, this may possibly explain the result in case No. 13.

The experiments show that, by the method adopted, the substance present in culture filtrates of *Streptococcus scarlatinæ* responsible for the characteristic skin reactions in susceptible human beings is present in the final product in a highly purified and concentrated form, and that the substance is neutralized by the antibody which is assumed to be present in the blood and tissues of persons immune to scarlet fever. The results of these experiments suggest that the substance present in the concentrated toxin which is lethal for rabbits may be the same as the substance which produces the typical skin reaction in susceptible human beings; they do not, however, furnish absolute proof of such identity, and further experiments are being undertaken to investigate this question.

7. *Variability in the Susceptibility of Rabbits to the Concentrated Scarlet Fever Toxin.*

During these experiments a certain amount of evidence has been obtained which suggests that some types of rabbits are more resistant than others to the action of the concentrated toxin. Ordinary grey or black rabbits, weighing from 1½ to 2½ kgm., as well as those of the Blue Beveren and Chinchilla breed, gave remarkably regular results. The supply of rabbits of these types failed for a few days and the work was continued using young Dutch rabbits. These were given doses of the toxin which had regularly killed larger rabbits of the types mentioned above: these young Dutch rabbits showed no symptoms and survived. It was thought at first that the concentrated toxin had lost potency, but when rabbits of the ordinary type became available again it was found that the preparation was just as toxic as before. A similar variation in susceptibility to the toxic action of filtrates from cultures of the hæmolytic streptococci group has been recorded by Clark and Felton (1918), Havens and Taylor (1921), and Zinnser, Parker and Kuttner (1920–21).

DISCUSSION.

The experiments thus far carried out have had a somewhat limited objective, viz. the preparation of toxin of sufficient potency to enable the standardization of scarlet fever antitoxin to be performed as a laboratory test on laboratory animals. It is considered that the results obtained are sufficiently promising to justify their publication in their present incomplete form. It is recognized that the method in its present form is largely empirical, due, in part, to the

fact that so little is known of the properties of the substance under investigation. Certain precautions have been taken which further research may show to be unnecessary. It has been assumed, for example, that the toxin is adversely affected by exposure to the action of acids, but the work of Watson and Wallace (1924) and the more recent work of Locke and Main (1928) suggests that diphtheria toxin may be exposed to acid conditions for several hours at low temperatures without danger, and if scarlet fever toxin should behave in a similar way, then the most difficult of the stages of the preparation of concentrated scarlet fever toxin by this method would be considerably simplified.

If these results can be confirmed by other workers using similar crude toxins, or crude toxins prepared by other methods, the preparation of concentrated scarlet fever toxin in sufficient amount to serve as a test toxin should not be difficult. Further, since the concentrated toxin can be dried without loss of specific properties, reconstruction can be effected by the solution of the dried toxin in much smaller volumes of solvent than was used for the purpose of these experiments, and thus a solution can be obtained containing twenty or more fatal doses per c.c.; moreover, such a solution would contain no more nitrogen and no more total solids than ordinary nutrient broth. Now the standard scarlet fever antitoxin recently prepared by the Hygienic Laboratory at Washington has been selected as a basis for further study of standardization problems by the Permanent Standards Commission of the Health Organization of the League of Nations, and is likely to be accepted as the standard for International use. By means of this standard antitoxin the "test dose" of the concentrated toxin can be ascertained, and thus the strength of scarlet fever antitoxins in terms of the Washington—and ultimately the International—standard can be determined. It would be necessary, of course, to determine whether concordant results are given by a method such as is outlined above, and by the methods now in use involving the carrying out of the titrations on the skin of susceptible human beings; in this connection the results of the experiments described in paragraph 6 above appear to be suggestive and promising, although, as Parish and Okell have pointed out, the difficulties of making such a comparison are considerable on account of the admittedly inaccurate nature of tests carried out on human beings; and in any case the ultimate value of a method of assay must be judged by the concordance between laboratory findings and therapeutic efficiency.

Although standardization of antitoxin has been the main and immediate concern of these experiments, there are many other purposes for which purified and concentrated streptococcal toxins may be usefully employed. Among these may be mentioned the use of such a product for the immunization of animals; from the results* obtained recently in this laboratory there are good grounds for believing that the use of purified antigens of this character would be of service in the production of antitoxic sera of high potency. Further, the preparation of the toxins of other pathogenic hæmolytic streptococci in the purified concentrated form and a comparison of their properties might contribute to the elucidation of the problems concerning the relationship of these closely related organisms.

* Communicated to the Pathological Society of Great Britain and Ireland, July 1st, 1927, and January 7th, 1928.

SUMMARY AND CONCLUSIONS.

1. Crude scarlet fever toxin, being the filtrate from cultures on tryptic digest broth of the Dochez strain of *Streptococcus scarlatinæ*, has been purified and concentrated by Walpole's method. During the process over 99% of the nitrogenous constituents of the original crude toxin were removed and the volume was reduced to one-twentieth in one experiment and to one-fortieth in another.

2. The solution of the purified toxin has been shown to possess the following properties:

(i) The solution is greenish-yellow to greenish-brown in colour, faintly opalescent, and almost free from visible particles.

(ii) The solution contains relatively small quantities of nitrogen; viz. 0·73 mgm. and 0·84 mgm. per c.c.

(iii) The solution is highly toxic for rabbits on intravenous injection. The minimum lethal dose of one concentrated toxin was about 0·5 c.c. (containing 0·36 mgm. nitrogen), and of the other about 0·2 c.c. (containing 0·17 mgm. nitrogen).

(iv) The toxin is apparently completely destroyed by heating to 100° C. for 1 hour, and is considerably weakened by heating to 56° C. for 1 hour.

(v) The toxin is neutralized by scarlet fever antitoxin. This neutralization effect can be demonstrated either by mixing the toxin and antitoxin *in vitro* and injecting the mixture intravenously, or by injecting the antitoxin some hours before administration of the toxin.

(vi) Two samples of puerperal streptococcus antitoxin also neutralized the concentrated toxin. One sample, a concentrated globulin preparation, proved to be as potent as the strongest scarlet fever antitoxin tested; the other sample was of feeble potency.

(vii) Three anti-streptococcus serums, known to have been prepared by the injection of emulsions of streptococci, made from strains other than the *Streptococcus scarlatinæ*, exhibited no protective or neutralizing action against the toxin.

(viii) Normal horse-serum was similarly inactive.

(ix) The solution of purified and concentrated toxin can be reduced to dryness without loss of specific properties; simple addition of water or salt solution to the dry preparation yields a solution indistinguishable, as regards appearance and biological properties, from the original solution. The dry preparation retained its specific properties unchanged and unimpaired for at least four months.

3. The active principle, present in crude filtrates from *Streptococcus scarlatinæ*, which is responsible for the characteristic skin reaction in susceptible human beings, is obtained in a highly purified and concentrated form in the final product obtained in these experiments. Comparative tests, carried out with a 20,000-fold dilution of concentrated scarlet fever toxin and a 1000-fold dilution of ordinary crude toxin, gave identical results in fourteen out of fifteen human beings examined; the group included both immune and susceptible individuals.

4. The results of these experiments suggest that it may be possible to evolve a method for the standardization of scarlet fever antitoxin, using concentrated scarlet fever toxin, prepared in the manner described, as test toxin and the rabbit as biological indicator.

5. Concentrated scarlet fever toxin may be of use for the immunization and hyperimmunization of animals, for the further study of the relationship of the different pathogenic hæmolytic streptococci, and for the study of scarlet fever susceptibility and immunity in man.

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ON THE CONCENTRATION AND PURIFICATION OF STREPTOCOCCAL TOXIN.

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Streptococci, long believed to produce no soluble toxin in artificial culture, are now known to be divisible into groups, certain members of which are definitely toxigenic (Dick, E. F., and Dick, G. H., 1924). Of these, the Dochez strain of *Streptococcus scarlatinæ* is perhaps the best recognized species. It has frequently been stated that the filtrates from the growth of this organism on liquid media are fatal for rabbits, but that the doses which must be injected to produce death are large, viz. from 10 to 40 c.c., and that the toxic effect of such filtrates can only be studied in susceptible human beings. The standardization of scarlet fever antitoxin accordingly presents difficulties, and the attempts which have been made to make use of laboratory animals have not up to the present been very successful (Kirkbride and Wheeler, 1926; Parish and Okell, 1928).

In the course of certain experiments of my own, which it is not necessary to describe in detail at this stage, it was found that the Dochez strain of the scarlet fever streptococcus, when grown under certain special conditions, uniformly yielded a toxin fatal for rabbits in doses of 2·5 to 10 c.c., the toxin being administered intravenously. The conditions adopted consisted of the cultivation of the organism in a special conical container, fresh medium being supplied continuously by a drip-feed, and the overflow, which contains the toxin, being collected in a special apparatus fitted with a filter. This method of cultivation has always yielded toxins of high potency. It was found subsequently, however, that 3-day cultures of the organism grown on the same medium in the ordinary way frequently, but not invariably, yielded toxins of equal potency. The factors determining the production of high-grade toxin have not been determined, but it has been found that 24-hour and 7-day cultures are always less potent than 3-day cultures; when grown for 1 or 7 days the filtrates have invariably failed to kill rabbits in doses of 10 c.c.

Preparation of Toxin.

Henry and Lewis (1925), and Mackie and McLachlin (1926), have shown that the active substance occurring in filtrates from the *Streptococcus scarlatinæ*, which causes the typical skin reaction in man, can be precipitated

from solution by means of alcohol. By means of alcohol precipitation it has been found possible to concentrate the toxins prepared in these experiments. Solutions of concentrated toxin have thus been obtained, which are fatal for rabbits sometimes in doses as small as 0·1 c.c. These concentrated solutions of toxin have been prepared in the following way:

One litre of peptone beef broth, free from serum, was inoculated with a 24-hour-old blood-agar slope culture. After 72 hours' incubation at 37° C., 50 c.c. were filtered through a Seitz filter, and the filtrate was tested for toxicity by the intravenous injection of 10 c.c., 5 c.c. and 2·5 c.c. quantities into rabbits. Two and a half litres of absolute alcohol were cooled to 0° C., and 500 c.c. of the culture were added slowly from a burette to the chilled alcohol. The container was maintained in ice and frequently shaken. The precipitate which formed adhered to the bottom and sides of the flask. The whole was allowed to stand in the refrigerator at 4° C. overnight, after which the supernatant fluid was decanted. A small amount of solid fails to adhere to the sides of the vessel, but it has been found that this contains little toxin. The flask was allowed to drain for half an hour and 25 c.c. of saline were then added. The solid separated quickly from the sides of the vessel and the suspension was transferred to a 100 c.c. flask. A further quantity of 25 c.c. of saline was then added to the large flask to remove the remaining traces of precipitate, and the whole 50 c.c. was then placed in a water-bath at 37° C. for 1½ hours and frequently shaken. The time and temperature are important, as it was found that the solutions obtained by extraction in a shaker for 3 hours at ordinary temperature were non-toxic. Finally, the saline extract was passed through a Seitz filter in order to remove the dead organisms and to ensure sterility.

This solution contains the toxin: it should be used immediately if possible, and must not be kept at refrigerator temperature. The concentrated toxins prepared in the manner described above have been found to be fatal for rabbits in doses as small as 0·1 c.c. The toxin is stable for at least 14 days if kept under absolute alcohol. Its stability in solution at room temperature has not been studied, though apparently it preserves its toxicity for 24 hours. When stored at 4° C. a precipitate is formed and the supernatant fluid is non-toxic. The toxin is destroyed by heating in an Arnold's steam sterilizer for half an hour.

Determination of the nitrogen content and total solids of the original and concentrated toxins has shown that 90% of the nitrogen and total solids are eliminated during the process. The strength of the concentrated toxin is more than 10 times that of the original filtrate, although the volume of the concentrated toxin is only one-tenth of the volume of the original culture. This may be due to the intimate adherence of some toxin to the organisms themselves, such toxin being removed and recovered in solution by the later manipulations.

Symptoms and Autopsy Findings.

A rabbit injected intravenously with a lethal dose of toxin remains well for 1 hour. It then becomes restless, breathes rapidly and passes liquid fæces.

It lies with eyes closed, occasionally stretching its legs and licking its abdomen. Death usually occurs within 24 hours, and if the animal survives for more than 24 hours it usually recovers, although death has occasionally occurred after periods as long as 5 days after injection. When death occurs within 6 hours the animal sometimes expires in convulsions.

Post-mortem, no changes are found at the site of injection in the ear. There is no free fluid in the serous cavities. The peritoneum is congested, and in female rabbits the uteri are particularly affected. The stomach shows marked hæmorrhagic erosion, which is shown in sections to affect the mucous membrane exclusively. This feature is more marked the longer the animal survives. In the case of one animal, which survived for 5 days, an early ulcer was found in the pylorus with inflammatory reaction. No changes were found in the small or large intestine. The kidneys show intense congestion, the medulla being deep red in colour and the cortico-medullary junction being very clearly defined. The urine contains no blood evident to the naked eye. Sections show degeneration of tubules with great congestion in glomeruli and tubules. In cases surviving for 5 days sections have shown cellular casts in the tubules and evidence of round-cell proliferation around the glomeruli. There is a serous exudate in Bowman's capsule and widespread necrosis of the tubular epithelium. Subserous hæmorrhages in the abdominal viscera and endocardial and pulmonary hæmorrhages have been noted in some cases. Sections of the brain in cases dying with convulsions or paralysis have not shown hæmorrhage. The spleen is very soft and shows much necrosis in sections. Heart-blood cultures have always been found to be sterile. The liver shows no changes, and no joint changes have been observed. Animals protected by antitoxin show no naked-eye or microscopic lesions when killed, but animals receiving sub-lethal doses show some or all of the conditions recorded above.

Intradermal injections into rabbits, mice and guinea-pigs produce no reaction. Mice injected intravenously with one-fifth of the dose lethal for rabbits have never shown signs of illness.

On two occasions small quantities of the toxin passed into the eye of the writer as a result of accident during injection. In spite of irrigation an intense conjunctivitis was set up, and there was general arthritic pain; vision was impaired for 24 hours. One of the samples of toxin which had been preserved at 4° C. for 48 hours, and had lost its toxicity for rabbits, was dropped into the conjunctival sac; slight conjunctivitis only followed. The toxin produces no effect on the human skin when dropped on it. A drop on the tongue gives a persistent burning sensation, but no other symptoms.

During the course of this work it has been found that rabbits vary in their susceptibility to the toxin, and this appears to be in some way related to the breed of rabbit. Havannah rabbits are relatively immune, while Chinchilla rabbits are susceptible, and whenever possible these latter have been used. Chinchilla rabbits, of from 2 to 3 kgm. in weight, were invariably used for the experiments on the neutralization by antitoxin. This type of rabbit is rather expensive, but dealers provide "Chinchilla type" rabbits at ordinary prices, and these have proved satisfactory.

Experiments with Specific Antitoxin, other Antisera and Normal Horse-serum.

In these experiments the dose of specific or other serum was injected into the marginal vein of one ear, and immediately afterwards the dose of toxin was injected intravenously into the other ear. In the case of each reagent the dose was injected in a volume of 5 c.c.

These experiments have shown that whereas concentrated scarlet fever antitoxin, kindly provided by Dr. R. A. O'Brien, of the Wellcome Physiological Research Laboratories, completely protected rabbits against the toxin, concentrated diphtheria and tetanus antitoxins and normal horse-serum were entirely without protective action. Anti-puerperal fever serum has sometimes protected animals and on other occasions has failed to do so.

Other Observations.

A sample of uninoculated broth of the type used in these experiments was precipitated in the same way as the streptococcus cultures and the precipitate so obtained re-dissolved. This material was tested on rabbits in doses of 10 to 20 c.c. and found to be non-toxic.

The solutions of concentrated toxin have been found to be free from hæmolysins.

Experiments have also been carried out to study the effects produced in rabbits by the repeated injection of sub-lethal doses of toxin, the results of which will form the subject of a later communication.

The variability, within very wide limits, of the minimum lethal dose of 3-day broth cultures of the Dochez strain of *Streptococcus scarlatinæ* requires further study. It has been found that repeated subculture of the strain every 12 hours on egg and blood media has no effect on the toxicogenic properties of the organism, and the same conclusion was drawn from experiments on the effect of animal passage. Growth of the organism in a flowing medium appears to yield high-grade toxin with regularity. Toxigenicity does not appear to be related to the question of the "rough" or "smooth" character of the strain, and no naked-eye difference is noticeable in cultures yielding high-grade and low-grade toxins. The only strains which, up to the present, have yielded toxin are the "Dochez" strain received from Dr. O'Brien and the "Sherman" strain from the Lister Institute. Other strains isolated from blood culture by the author have not yielded toxin; these strains differed culturally from the "Dochez" strain.

The effect of these high-grade toxins on antitoxin production has not yet been investigated. From the protocols which follow, however, it will be seen that the antitoxic sera at present available are of remarkably high efficiency, doses as small as 0·025 c.c. being sufficient to save the life of rabbits injected simultaneously with 10 minimum lethal doses of the toxin. Animals receiving this small amount, however, are not fully protected against the toxic effects of the preparation, since they become ill, refuse to eat and suffer from diarrhœa. 0·2 c.c. doses of the antitoxin do not entirely suppress all symptoms, although the animals survive. Larger doses afford complete protection. In determining the strength of an antitoxin, therefore, it may be desirable to take into

account not only the death or survival of the animal, but also the occurrence or absence of diarrhœa—always a well-marked symptom.

SUMMARY.

1. Filtrates from cultures of the Dochez strain of *Streptococcus scarlatinæ* have been prepared which are fatal for rabbits in doses of 2·5 c.c. to 10 c.c.

2. Breeds of rabbits vary in their susceptibility to this toxin; Chinchilla rabbits are uniformly susceptible.

3. By means of precipitation of whole cultures with ice-cold absolute alcohol, concentration and purification of toxin may be effected. Such a concentrated toxin is sometimes lethal in doses as small as 0·1 c.c.

4. Complete protection is afforded to rabbits against large doses of toxin by streptococcal antitoxic sera. Tetanus and diphtheria antitoxins and normal horse-serum are without effect.

5. By means of this toxin the strength of antitoxic sera may be estimated, using the rabbit as test animal.

My thanks are due to Prof. H. MacLean and Sir Cuthbert Wallace for facilities in carrying out this work; to Prof. L. S. Dudgeon for advice and criticism; to Dr. R. O'Brien and his colleagues for the antisera and strain of organism used, and for much useful advice; and to my assistant, Mr. P. Carter, for his valuable help.

In particular, Dr. P. Hartley, whose assistance I sought on learning of his work on the same subject, has afforded me most generous help in suggesting certain lines of experimental inquiry.

PROTOCOLS.

(All injections were by intravenous route.)

EXPERIMENT 1.—*Results obtained with the Filtrate from a 72-hour Culture of* Streptococcus scarlatinæ *(Dochez Strain), Unconcentrated.*

Chinchilla rabbits used.

Rabbit.	Injection given.	Result.
A	10 c.c. toxin	Died 24 hours.
B	5 „ „	„ 24 „
C	5 „ „	„ 24 „
D	5 „ „	„ 45 „
E	2·5 „ „	„ 5 days.
F	1 „ „	Diarrhœa; recovered.
G	10 „ toxin and 5 c.c. concentrated scarlet fever antitoxin	No symptoms; recovered.
H	10 „ „ 5 „ ditto	„ „
I	10 „ „ 5 „ concentrated tetanus antitoxin	Died 24 hours.
J	10 „ „ 5 „ concentrated diphtheria antitoxin	„ 24 „
K	10 „ „ 10 „ normal horse-serum	„ 24 „
L	10 „ toxin heated for 30 min. in Arnold's steam sterilizer	No symptoms; recovered.

In this experiment the toxin was prepared from a culture grown in flowing medium. The same result was obtained from four other similar experiments, save that the rabbit receiving 2·5 c.c. of toxin died within 24 hours. Smaller doses were never fatal; no rabbit receiving specific antitoxin died.

EXPERIMENT 2.—*Results obtained with the Filtrate from a 72-hour-old Culture Grown in the Normal Way (Unconcentrated).*

A mixed breed of rabbit used.

Rabbit.	Injection given.	Result.
A	10 c.c. toxin	Died 24 hours.
B	10 „ „	„ 12 „
C	10 „ „	Diarrhœa; recovered.
D	5 „ „	Died 12 hours.
E	2·5 „ „	„ 48 „
F	10 „ toxin and 5 c.c. concentrated scarlet fever antitoxin	No symptoms; recovered.
G	10 „ „ 5 „ concentrated diphtheria antitoxin	Died 24 hours.

This toxin was concentrated by alcohol precipitation and the product used for Experiment 3.

EXPERIMENT 3.—*Results obtained with Precipitated Toxin Re-dissolved by Warming to 37° C.*

Mixed breed of rabbits used.

Rabbit.	Injection given.	Result.
A	5 c.c. toxin	Died 12 hours.
B	2 „ „	„ 12 „
C	1 „ „	„ 12 „
D	0·5 „ „	„ 12 „
E	0·2 „ „	Ill; recovered.
F	0·1 „ „	Died 12 hours.
G	0·05 „ „	No symptoms; survived.
H	5 „ „ and 5 c.c. concentrated scarlet fever antitoxin	„ „
I	Precipitate from 200 c.c. of beef-broth re-dissolved in 20 c.c. saline	„ „

This experiment was repeated with similar results.

EXPERIMENT 4.

Chinchilla rabbits were used. The concentrated toxin was prepared from a filtrate which was lethal for rabbits in doses of 10 c.c., and the alcohol precipitate was re-dissolved in saline by warming at 37° C. for 1½ hours.

Rabbit.	Injection given.	Result.
A	2 c.c. toxin	Died 24 hours.
B	1 „ „	„ 72 „
C	0·5 „ „	„ 72 „
D	0·25 „ „	Diarrhœa; recovered.
E	5 „ toxin and 5 c.c. concentrated diphtheria antitoxin	Died 24 hours.

EXPERIMENT 4—*continued.*

Rabbit.	Injection given.	Result.
F	5 c.c. toxin and 5 c.c. concentrated tetanus antitoxin	Died 24 hours.
G	5 „ „ 0·0125 c.c. concentrated scarlet fever antitoxin	„ 24 „
H	5 „ „ 0·0125 „ ditto	„ 48 „
I & J	5 „ „ 0·025 „ „	Diarrhœa; recovered.
K	5 „ „ 0·05 „ „	„ „
L	5 „ „ 0·1 „ „	„ „
M	5 „ „ 0·2 „ „	„ „
N	5 „ „ 0·4 „ „	No symptoms; recovered.
O	5 „ „ 1 „ „	„ „
P	5 „ „ 5 „ „	„ „
Q	5 „ „ 10 „ „	„ „

This experiment was repeated, each dose being injected into two rabbits, with similar results, save that one animal receiving 0·025 c.c. antitoxin died.

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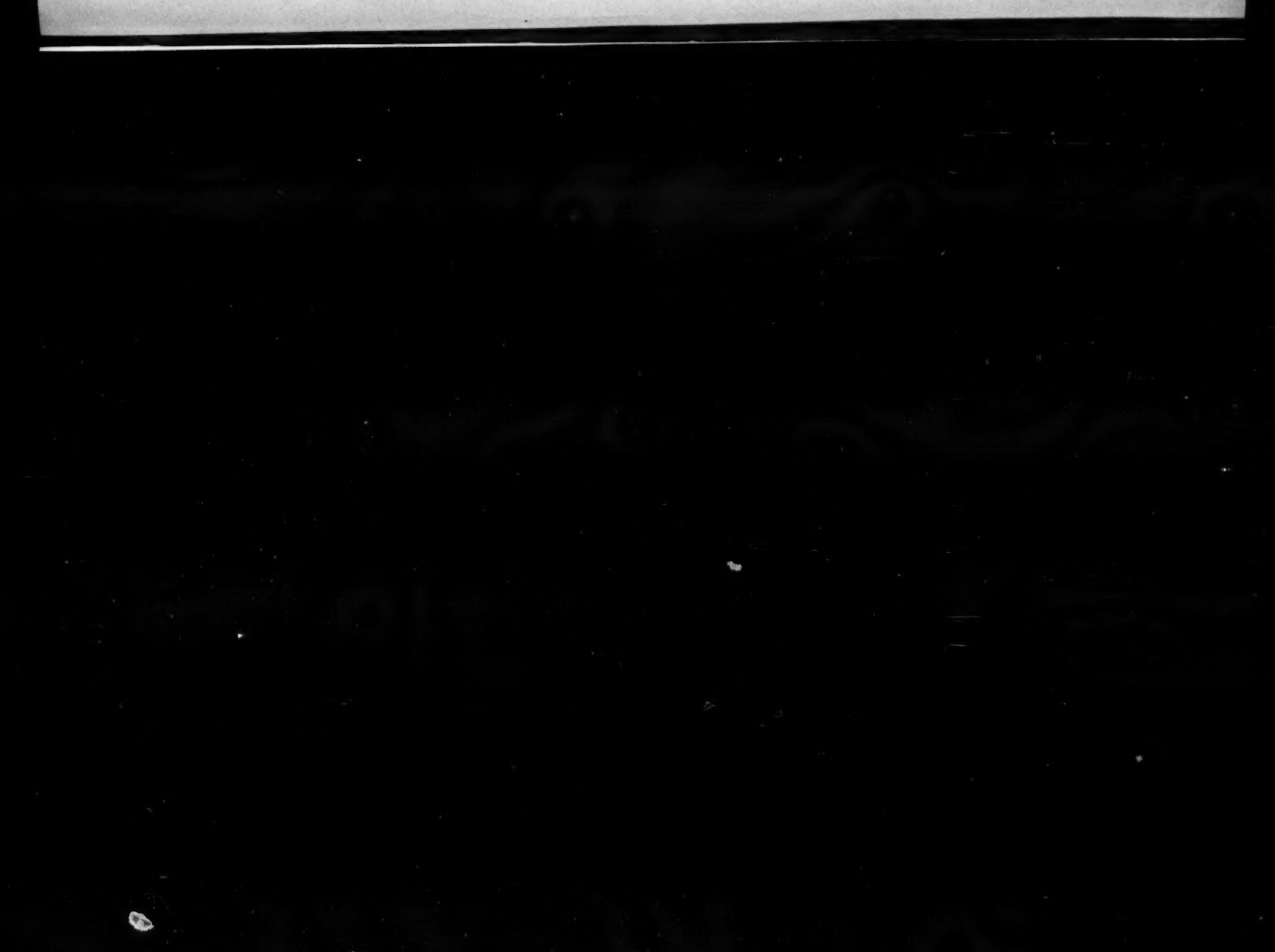

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